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Review

7-Ketocholesterol as marker of cholesterol oxidation in model and food systems: When and how

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ABSTRACT

Cholesterol can undergo oxidation through enzymatic or chemical mechanisms, generating a wide range of oxidation products (COPs) with adverse biological effects. COPs are characterized by different functional groups and are produced in different ratios or amounts, depending on the treatment and storage conditions. To follow the cholesterol oxidation process, 7-ketocholesterol (7-KC) has been often used as an oxidation marker in both model and food systems, since it is easily formed and is one of the most representative ring COPs. However, 7-KC does not always rise with increasing time/temperature conditions, especially in complex systems and high-protein or extensively processed foods. The following review provides a critical picture of the utilization of 7-KC as a cholesterol oxidation marker in model and food systems, focusing on the possible causes and effects of the different behaviours and trends, as well as on the advantages and disadvantages of using 7-KC when the extent of cholesterol oxidation is to be assessed.

Contents

1. Introduction

Cholesterol is a monounsaturated constituent of cell membranes and is involved in their permeability and fluidity. Due to the presence of a double bond (carbon 5), a wide range of cholesterol oxidation products (COPs) can be produced endogenously or exogenously through different reaction mechanisms and pathways (chemical, photosensitized and enzymatic oxidation). Metabolic dysfunctions or the frequent consumption of COP-containing foods can be potentially harmful to health, since COPs can have negative biological actions (atherogenic, cytotoxic, mutagenic, apoptotic and carcinogenic effects), and are likely to be involved in several chronic and degenerative diseases, as well as in disturbances of cell functionality and lipid metabolism [1–3]. Several reviews have

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discussed the routes of oxysterol formation and their major biological effects [1–6].

The first products of cholesterol oxidation are hydroperoxides (ROOH, mainly in positions 5 and 7), which can undergo dismutation that generates 7α-hydroxycholesterol (7α-HC) and 7β-hydroxycholesterol (7β-HC), together with 7-ketocholesterol (7-KC). The formation of epoxy derivatives (5α,6α-epoxycholesterol (α-EC) and 5β,6β-epoxycholesterol (β-EC)) occurs via a bimolecular reaction mechanism through the interaction of a hydroperoxy radical and cholesterol [4]. In the presence of water and acidic conditions, epoxy derivatives in turn can experience oxirane-ring opening and thus produce cholestanetriol (CT). Generally, ring COPs tend to be formed non-enzymatically, whereas side-chain oxysterols usually have an enzymatic origin, except for 25-hydroxycholesterol (25-HC) and 7α-HC, which can be produced by both routes [2].

7-KC has been often used as a marker of cholesterol oxidation in both model and food systems, since it is easily formed and is one of the most representative oxysterols (>30% of total COPs) [4,7]. Several kinetic models have been proposed for cholesterol oxidation in food and model systems [6]. However, 7-KC does not always rise with increasing time/temperature conditions, especially in complex systems and high-protein or highly processed foods. The stability of cholesterol in complex mixtures is influenced by interactions among these components and/or their decomposition products. Moreover, the large number of molecules generated through oxidation, together with the presence or absence of antioxidants and proxidants and reactions with other macromolecules (proteins, carbohydrates, lipids) cannot only shift the cholesterol oxidation rate, but also modify the shape of the oxidation curve itself and the relative oxysterol distribution.

This review aims to provide a critical view of the utilization of 7-KC as a marker of cholesterol oxidation in model and food systems, focusing on the possible causes and effects of the different behaviours and trends, as well as on the advantages and disadvantages of this marker choice.

2. Model systems

In most model system studies, 7-KC has been reported as the most abundant COP (30–70% of total COPs). Cholesterol degradation follows a first order kinetic model if cholesterol is present as a solid or in solution and is subjected to either thermoxidation or photoxidation [6]. A first order kinetic model has also been suggested for 7-KC formation when cholesterol was thermoxidized in the solid state at 150 °C [8]. Cholesterol has proven to be virtually stable during heating at 100 °C for 24 h, but is unstable at temperatures above 120 °C [9]. When cholesterol was heated at 140 °C, only 7-hydroperoxycholesterol was formed until 213 s, whereas 7-KC, 7α-HC and 7β-HC were generated between 213 and 593 s; this was subsequently followed by the formation of α-EC and β-EC [10]. Cholesterol degradation at 140 °C occurs slowly, as this temperature is below its melting point [11]. However, this behaviour was not observed at higher temperatures (180 and 220 °C), where all these five COPs were already present at the first sampling time point [10]. At these high temperatures, cholesterol not only generates the most common COPs, but is also involved in the formation of dehydration compounds, oligomers and volatile compounds [10,12]. Moreover, under these conditions, 7-KC can also dehydrate and give rise to cholesta-3,5-dien-7-one. The latter can also be generated during photoxidation at room temperature (RT), probably due to 7-KC dehydration through energy released during light exposure [13].

The physical state of the model system (liquid or powder) can also affect the rate of 7-KC formation. In model food powders [14], surface composition and structure greatly influenced the cholesterol oxidation rate. Although α-EC was always the most abundant COP at the beginning of degradation (regardless of model food powder composition), 7-KC and 7β-HC became the predominant oxysterols after 6-month storage in darkness at RT [14]. In aqueous model systems, the pH value can also modify the trend of cholesterol oxidation, since CT formation is greatly favoured under acidic conditions. The pH conditions can also affect the activity of proxidant or antioxidant compounds, since they can modify the molecule chemical properties (protonated or reduced forms). Ionizing radiation can also generate COPs in aqueous systems; although the products are similar to those formed by autoxidation, they present different relative amounts [15]. When exposed to gamma radiation, the main COPs are 7-KC and the 5,6-epoxy derivatives.

On evaluating the influence of the degree of unsaturation of different triacylglycerols (TAG) upon cholesterol thermoxidation at 180 °C, 7-KC was found to be the most abundant COP, except in the presence of trilinolenin where 7β-HC was predominant [16]. Furthermore, when cholesterol was heated alone or with tristearin, a decrease in 7-KC was noted after 20 and 120 min, respectively, yielding an overall decrease in total COPs. Other authors have noted similar 7-KC behaviour under analogous thermoxidation conditions, but the decline in 7-KC was faster probably due to the different heating transfer modalities and sample amount:vial volume ratios involved [17]. Cholesterol degraded more rapidly when it was heated alone than in the presence of TAG, which could be ascribed to a dilution effect, TAG physical protection against oxygen contact and/or TAG competition for oxidation. TAG physical arrangement and molecular hindrance, as well as chemical group interaction and viscosity increase due to polymerization, might also have influenced these trends. Another research group [18] incubated cholesterol with fish oil TAGs (with different degrees of unsaturation) at RT; although 7-KC rose continuously during the whole 39-day storage period, both 7β-HC and β-EC became the most abundant COPs after 27 days. In an analogous study [19], 7-KC was found to be a reliable marker in all cholesterol-TAGs model systems oxidized at 100 °C for 24 h, but dropped after 12 h in the most unsaturated TAG systems (linseed and sardine oils).

When cholesterol was oxidized in the presence of superoxide anion, water and hydrogen peroxide, α-tocopherol, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) markedly retarded the formation of 7-KC [20]. A protective role of carotenoids against 7-KC formation in solution has been also observed [21]. When evaluating the combined effect of riboflavin and fatty acid methyl esters (FAME) upon cholesterol photoxidation (25 °C for 28 days), the most relevant COPs were either 7-KC or β-EC, depending on the amount of antioxidant and the type of FAME involved [13]. Moreover, 7-KC tended to decrease after 14 days of storage in all the trials, except when cholesterol alone was photoxidized. In another study [22], cholesterol was thermally oxidized at 140 °C in the presence of stearylamine, yielding epoxy and triol derivatives as the main oxysterols. Stearylamine was able to reduce both the oxidation and degradation rates of cholesterol, which could be due to the formation of antioxidant compounds through the reaction between amines (primary and secondary) and alkenals or epoxyalkenals [23].

3. Food

The behaviour of COPs and 7-KC in food greatly depends on the type of food matrix involved (physicochemical and enzymatic characteristics), as well as on the processing and storage conditions to which they are subjected. Therefore, the suitability and reliability of 7-KC as a marker of cholesterol oxidation should be assessed according to the food matrix involved.

3.1. Egg products and egg-based products

The great consumption of industrialized egg-containing foods (such as bakery products, salad dressings and pasta) has led to

the production of egg powder, which is characterized by its longer shelf-life. Spray-dried egg has been one of the most widely used models for COP formation studies in the past 25 years, focusing on the storage and drying processes. Initial studies dealt with the effect of the drying process upon COP formation, and showed that, in general, COP and 7-KC formation seems to be much greater in direct fired dryers (flame heating) than in indirect ones (electrical heating). This fact was attributed to the formation of nitrogen oxides (NO_x = NO + NO_2) in the flame-heated air.

The modifications in NO_x levels introduced in the gas stream and the outlet temperatures of the spray dryer showed that, within each range of NO_x, the levels of 7-KC (and total COPs) were significantly higher in products dried at the highest temperature, the most abundant oxide in all cases being β-EC [24]. No significant differences in 7-KC levels were detected among three different conditions of inlet and outlet temperatures, in contrast to the trend noted for the other oxysterols [25]. Moreover, levels of COPs in egg samples increased during spray-drying, especially for 7-KC which represented about 50% of total COPs, but no β-EC was detected.

Studies related to the storage of egg powders at RT reported that, in general, 7-KC seemed to accumulate (including under vacuum and dark storage conditions), even after a non-increase lag period. In studies under accelerated storage, a slight decrease has been noted right after 7-KC accumulation; this drop could be attributed to reaction with amino acids/proteins or Maillard reaction products (MRP), or to dehydration as discussed above. It also must be considered that, in many studies, β-EC was the predominant oxide, despite the increase in 7-KC levels. In this sense, 7-KC levels increased (always <15% of relative abundance) during storage (up to 6 months) of egg powder, whereas β-EC was the most abundant oxysterol (approximately 50% of total COPs) [26,27].

Longer periods of storage of whole egg powder at RT have been also tested [28–31]. Wahle et al. [28] reported that 7-KC increased after a lag period of 2–4 months and reached a maximum level at 12 months of storage in samples stored under daylight and exposed to air. After 18 months, its concentration decreased, even though it was the most abundant COP at any time. Similar behaviour was described for 7β-HC and epoxy derivatives (the exact isomer not being indicated) [28]. In contrast, other studies have described a similar trend of 7-KC accumulation during storage (12 months, darkness and/or vacuum) [29–31]. However, β-EC and 7β-HC were the main oxysterols detected (up to 42% of total COPs), 7-KC being present at lower levels (10–15%), probably because of the different storage conditions involved.

Elevated temperature or accelerated storage (90 °C for 24 h or 60 °C for 28 days in the presence of Cu^{2+}) increased the COP contents and stimulated scission of 7-hydroperoxides of cholesterol, generating a prevalence of 7-KC over 7-hydroxy derivatives. These conditions seemed to also promote conversion of α-EC into CT, whereas epoxy derivatives tended to accumulate at RT storage [32,33]. Therefore, different mechanisms of oxidation may account for differences in the relative abundance of various COPs generated during RT storage with respect to accelerated storage.

The protective effect of antioxidants during the storage of egg products has not been established, even though the addition of tocopherols and BHA seem to inhibit C-7 oxides formation [32,33].

In eggs enriched with polyunsaturated fatty acids, the increase in unsaturation makes them susceptible to oxidation during processing (including cooking) and storage. Accumulation of 7-KC during storage of enriched eggs has been reported, even though the addition of antioxidants proved to inhibit its formation [34,35].

Regarding egg-based products, the amount of COPs in cookies increased during storage; however, only 7-KC appeared to decrease with ageing, despite the fact that it was initially the predominant oxide (from 54% to 20% after 12 months) [31]. The accumulation of 7-KC has been described for dried egg pasta in two studies that reported analogous values at zero time and after 12 months of storage [36,37]. However, prolonged storage (18 months) induced a new increase in 7-KC levels [36]. In freshly made mayonnaise [38], 7-KC was the only oxysterol detected, and it accumulated during storage regardless of the temperature conditions (4 and 25 °C). However, at the end of storage, 25-HC was the most abundant COP, and similar levels of 7-KC (24% of abundance) and hydroxy derivatives were reported.

3.2. Fish and seafood

Noticeable differences in cholesterol oxidation rate and single COPs trends have been observed in fish and seafood, depending on the processing and storage parameters. When whole sardines were stored at 4 °C for 4 h under light exposure and in the dark [39], the most abundant COP in untreated samples was 7-KC, followed by β-CE and 7β-HC. This confirms that 7-KC can be employed as a suitable marker of cholesterol oxidation in raw muscle foods. The formation of relevant amounts of epoxy derivatives might be partly due to the interaction of sterols with hydrogen peroxide released by endogenous microbial enzymes in muscle tissues [40]. 7-KC increased during light exposure due to large hydroperoxide breakdown, but it showed a bell-shape behaviour when kept in darkness. This could be ascribed to 7-KC decomposition and/or interaction with amino compounds or other components, thus generating derivatives that are not detectable under the analytical conditions used [41].

Fresh fillets of Atlantic hake were stored at −18 °C for 120 days, and the formation of COPs during storage and subsequent grilling was evaluated [42]. Fresh hake showed low COP levels, but a significant increase in COPs was observed during frozen storage and after grilling. The main cholesterol oxides were uncommon side-chain oxysterols and 7-KC. A feasible explanation for such particular behaviour could be the influence of the physical state of cholesterol upon COP formation [43]; in its crystalline state, the 3-hydroxyl groups are juxtapositioned and the side chain is exposed to reagent attack [44]. After 120 days of frozen storage, 7-KC became the main oxidation product [42].

The effect of grilling on the formation of oxysterols in boiled and dried anchovy has been investigated [45]. In this context, 7β-HC and β-EC were the main COPs identified. The increase in 7-KC was enhanced during heating (<6 min); however, further grilling time did not affect the level of 7-KC, even though it significantly decreased total COPs. During grilling, peroxidised lipids and cholesterol are thermally decomposed, and pyrolysis of oxysterols also occurs.

In cooked rainbow trout previously supplemented with dietary α-tocopherol and/or subjected to surface application of oleoresin rosemary, β-EC and 7β-HC were the major COPs [46]. Although 7-KC increased during refrigerated storage, the formation of COPs was reduced by the presence of the antioxidants, and their relative distribution did not change.

The formation of major oxysterols was monitored in dried salted shrimp during cooking, sun drying and storage [47]. COPs showed a dramatic increase during sun drying, and rose further during storage. Although 7-KC increased during the whole drying period, it was not the major COP, 7-hydroxy and epoxy derivatives being the predominant COPs. This indicates that 7-KC dehydration may have occurred, and that the equilibrium of the dismutation reaction of hydroperoxides might have favoured their conversion to hydroxyl derivatives. In contrast to sun drying, 7-KC was formed in amounts similar to 7β-HC and 7α-HC during storage.

3.3. Meat and meat products

COPs in raw meats are usually present in low amounts, but their concentrations tend to increase dramatically after exposure to proxidant agents such as light [48–51]. Photoxidation of turkey, beef and horse meat has been studied [49–51] under different atmospheric compositions, lighting conditions and wrapping properties. In general, COPs displayed an increasing or bell-shaped trend during light exposure, due to fast hydroperoxide depletion. 7-KC was usually the most abundant oxysterol (about 30% of total COPs) in photoxidized meat. COP decline could be ascribed to decomposition and/or reaction with other molecules (such as proteins) [41,52] that might generate non-detectable compounds.

During storage of fresh meat and meat products, 7-KC tends to increase and usually becomes the most relevant COP, regardless of dietary supplementation and packaging conditions [53–57].

Cholesterol oxidation is critical in sliced meat products, due to the very large surface-to-volume ratio. When lipid oxidation was assessed in Milano-type fermented sausages as related to packaging conditions and extended storage under fluorescent light [58], COPs increased significantly during storage, and 7-KC was the most abundant oxide.

In irradiated and stored meat products, the effect of radiation on cholesterol oxidation could differ depending on the type of product [59–60]. In most cases, COPs increased after irradiation and during storage, 7β-HC, epoxy derivatives and 7-KC being the major COPs. Similar results were found in irradiated raw and cooked chicken meats with different packaging and storage times [61–62].

Treatment of mechanically deboned turkey meat by high hydrostatic pressure also induces the formation of COPs, in particular 7-KC [63].

In marinated meat products, the best markers of cholesterol oxidation were β-EC and 7-KC, but their contents were affected by the antioxidant properties of MRP present in the marinade [64].

Cooking usually favours the formation of COPs, but their relative composition and accumulation greatly depend on the initial oxidative status of the meat, the type of meat, and the cooking conditions [41,54,65–66]. Generally, 7-KC is not the most abundant oxysterol, since during cooking its breakdown rate seems to be greater than its formation rate, thus giving rise to undetected compounds. In turn, 7-KC could also interact with other compounds (such as proteins, peptides, or free amino acids), and consequently form Schiff bases [41,67]. As already mentioned for fish, extensive cooking at high temperatures may also lead to cholesterol and COP dehydration and pyrolysis.

Several studies have reported that 7-KC is a reliable cholesterol oxidation marker for cooked meat subjected to storage [54,57,65,68–70]. However, in fried meat products, 7β-HC and β-EC are generally the main COPs detected [71].

3.4. Milk and dairy products

There are few studies in the literature on COP profiles in fresh milk, but more references can be found about milk powder. In general, fresh milk and dairy products contain little or no COPs. However, the formation of COPs in these food products is particularly favoured in dehydrated products [72].

The UHT treatment of milk (bovine and caprine) significantly increased 7-KC levels as compared to raw samples [73]. Throughout storage (6 months at 4 °C or 20 °C), 7-KC was found to accumulate, but its contribution was reduced by half due to the greater presence of 7α-HC [73]. Moreover, UHT processing initiated oxidation at the side chain of cholesterol, producing a significant increase in 25-HC levels. The contribution of CT was very low in all treatments, indicating slow conversion of epoxycholesterols to this derivative. Microwave and conventional heating processes could similarly contribute to the formation of COPs, without a significant increase in 7-KC [74]; in these cases, 7β-HC was the most abundant oxysterol.

While COPs were not detected in freshly prepared milk powders, aged samples contained varying amounts, depending on the drying technology used, and the packaging and storage conditions. In this sense, cholesterol oxidation is minimized by using drying processes that generate low levels of NO_x, at reduced temperatures and with the exclusion of oxygen [75]. Several studies demonstrated that the most abundant COPs in stored milk powders are 7-KC, 7-hydroxy and epoxy derivatives. Although a 7-KC accumulation trend can be noted during storage, systematic storage studies of milk powders have not been performed as for egg products. 7-KC and 7β-HC were the main oxides detected in whole milk powder (WMP) and skimmed milk powder (SMP) samples during 12-month storage (at 32 and 55 °C) [76]. The maximum level was reached after 9 months and was maintained until the end of storage. The relative abundance of 7-KC ranged from 22% to 63% of total COPs, but its content was up to 10-fold higher in WMP than in SMP. In samples stored at 55 °C under nitrogen, an increase in CT and α-EC levels was also observed; this seems to indicate that a double-oxidation mechanism, via ground-state dioxygen and hydroxyperoxide-induced free radicals, may have occurred [76].

Large differences in 7-KC behaviour have been reported during the storage, processing or heating of dairy products (such as butter and cheese). Although 7-KC was found at trace levels or was not detected in fresh butter samples [75,77–79], it tended to accumulate during storage. Epoxy derivatives were also likely to increase under non-chilled conditions and prolonged storage. Low amounts of 7-KC (<32% of total COPs) were detected in butter after two weeks of storage (at 4 and 16 °C), remaining stable even after 6 months [77]. At the end of storage, α-EC was the most abundant product at 4 °C and 16 °C [77], and both epoxycholesterols accumulated in amounts twice as much as that of 7-KC (14% of abundance) after 4 months [75].

This trend was not confirmed in another work [80] in which 7-KC was found to be the main oxysterol (50–98%) in butter and dairy spread stored under similar conditions (4 and 20 °C) after three months. 7-Hydroxy derivatives were the second most abundant COP class. Cholesterol epoxides and CT accumulated only in the dairy spread samples, though. Other studies evaluating butter storage under different lighting conditions (daylight, fluorescent, UV) did not obtain conclusive results [72].

To accumulate 7-KC in butter samples, it was necessary to apply temperatures over 170 °C. Under these conditions, 7-KC became the most relevant oxide (35–40% of total COPs), followed by β-EC and 7-hydroxy derivatives in the same proportion [75]. Different amounts and ratios of the same oxysterols (α-EC > 7β-HC > 7-KC) were detected after subjecting cow and buffalo ghee to different thermal treatments [78]. In another study [79], COP levels (β-EC > 7-KC > 7β-HC > α-EC) rose in butter with increasing processing time and temperature.

Mild storage conditions (4 °C) do not seem to promote 7-KC accumulation [77,81] in cheese samples. However, its levels increased with longer (>7 months) or more aggressive storage conditions (RT, light-exposed, grated samples) [75,77,82]. Other oxysterols (α-EC and 7α-HC) showed the same trend. In this sense, the accelerated storage of feta cheese (30 days, 4 °C, soaked in brine and exposed to air) markedly elevated the 7-KC content (40-fold), and produced a greater accumulation of epoxy derivatives than hydroxyl derivatives [81].

Low 7-KC content has been reported in human milk [83] and infant milk cereals [84]. In the latter, no significant variations were detected during storage at RT for 9 months (0.6–1.5% of abundance).

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