Review

Cholesterol and its oxidation products: occurrence and analysis in milk and milk products

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Abstract

The cholesterol is one of the important components of biological membranes. It is associated with milk fat in milk and milk products. Cholesterol present in animal origin foods undergoes autoxidation during processing as well as during storage yielding toxic products commonly known as cholesterol oxidation products (COPs). The COPs are significantly affected the human health such as atherosclerosis, inflammation, cancer, neurodegenerative diseases etc. Various methods are reported in literature for determination of cholesterol and its oxidation products in milk and milk products.

1   Introduction

The name cholesterol was derived from two Greek words *chole* (means bile) and *stereos* (means solid). Cholesterol is one of the components of biological membranes. Cholesterol is found in all body tissues especially the brain and spinal cord (Raguz *et al.,* 2011). It is also found in many of the animal food products like eggs, dairy products, poultry, fish, lard and other fats.

Cholesterol, an unsaturated alcohol, undergoes oxidation resulting in the formation of toxic products. Cholesterol undergoes auto-oxidation, photo-oxidation and enzymatic-oxidation, producing relevant hydroperoxides (Ubhayasekera *et al.,* 2004). Cholesterol oxidation products (COPs) are similar to cholesterol, which contain an additional functional group, such as a hydroxyl, ketone or an epoxide group. The major factors that influence COPs formation during food processing or storage are heat, pH, light, oxygen, water activity and the presence of unsaturated fatty acids. Various analytical approaches have been described in the literature for example, colorimetric, gravimetric, chromatographic, infrared, and so forth for estimation of cholesterol in dairy and food products. For cholesterol estimation in dairy products, two main methods have been developed viz; direct and indirect. In the direct method, color is developed in anhydrous milk fat (i.e. ghee) dissolved in chloroform to a suitable dilution (Bindal and Jain 1973) whereas, in the indirect method unsaponifiable matter is used for color development (Pantulu and Murthy 1982). Other methods like Fourier transformed infrared (FTIR) spectroscopy, high-performance liquid chromatography (HPLC) are also reported.

2   Structure and properties of cholesterol

Chemically, cholesterol is a fat-like compound, basically, an alcohol which, in its pure form appears as flakes. It is composed of 27-carbon atoms which form three fused cyclohexane (6-carbon) rings, a cyclo-pentane (5 carbons) ring and a side chain of eight carbon atoms. Its molecular weight is 386.66 and the molecular formula is $C_{27}H_{46}O$. The melting point of cholesterol is $148.50^{\circ}C$. Cholesterol contains the polar hydroxyl group at C-3, which gives cholesterol a slightly hydrophilic nature, can be esterified to a fatty acid, producing cholesterol ester. Cholesterol is insoluble in water, sparingly soluble in cold alcohol or petroleum ether, and completely soluble in hot alcohol and in most other organic solvents.

All the 27 carbon atoms of cholesterol are derived from acetyl CoA through the 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) pathway. First of all formation of mevalonic acid from three molecules of acetyl CoA then biosynthesis of squalene from six molecules of mevalonic acid through a series of phosphorylated intermediates. Subsequently the biosynthesis of lanosterol from squalene via cyclization of 2, 3-epoxysqualene, and modification of lanosterol to produce cholesterol (Li and Parish 1998).

3 Significance of cholesterol

Cholesterol is more abundant in tissues having densely packed membranes, like the liver, spinal cord, and brain. There are mainly two types of cholesterol: high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol. If cholesterol is high, it builds upon the walls of the arteries leading to a heart attack or a stroke. Cholesterol is a major determinant of membrane fluidity due to its hydrophobic and hydrophilic regions. Cholesterol is a structural component of the cell membranes. Approximately 80% of cholesterol is used by the liver to form the bile acids. Cholic acid is the most abundant bile acid in human bile. Eventually, bile acids get coupled to glycine and taurine to form salts of these substances which are water soluble and act as powerful emulsifiers, important in the digestion of fat. It is a structural component of the cell membranes particularly skin and the myelin sheath. It is a chemical ancestor of the hormones like progesterone, cortisone and estrogen containing 21, 19, and 18 carbon atoms, respectively. These hormones are found in the ovaries, testis and adrenal gland. It is a precursor for the synthesis of vitamin D (Makwana 2007).

4 Cholesterol in milk and milk products

Cholesterol is rich in animal food products like milk and milk products. Cholesterol accounts for 0.25-0.40% of the total lipids in milk (Jenness and Patton 1959). In milk, it is present in the fat globule membrane (FGM), in the fat core and in association with milk protein particularly in skimmed milk (Schlimme and Kiel 1989). Any process disrupting the membrane structure will result in the transfer of cholesterol along with ruptured membrane material to the aqueous phase. Tylkin *et al.* (1975) reported 9 times higher cholesterol per gram fat in buttermilk than that in butter. Vyshemirskii *et al.* (1977) reported that 80-90% cholesterol initially present in cream passed into butter and 10-20% to buttermilk. Bindal and Jain (1973) estimated free and esterified cholesterol in desi ghee and reported their contents as 0.288 and 0.038%, 0.214% and 0.056% in cow and buffalo ghee, respectively. The cholesterol in cheese was found to contain 69.3 mg/100 g of cheese (Fuke and Matsuoka 1974). The cholesterol content in unsalted butter and 10% cream were reported to contain 244 and 31.4 mg / 100g of product, respectively (Aristova and Bekhova 1976). Arul *et al.* (1988) studied the distribution of cholesterol in various milk fat fractions viz., solid fraction (melting point 39°C), semisolid fraction (melting point 21°C) and liquid fraction (melting point. 12°C) and reported that 80% of the total cholesterol content was present in the liquid fraction of the milk fat. Bindal and Jain (1973) estimated free and esterified cholesterol in desi ghee and reported their contents as 0.288 and 0.038%, 0.214 and 0.056% in cow and buffalo ghee, respectively. The cheese was found to contain 52.3 to 76.6 (average 69.3) mg of cholesterol per 100 g of cheese and 198 to 298 (average 273) mg of cholesterol per 100 g of cheese fat (Fuke and Matsuoka 1974). Unsalted butter and 10% cream were reported to contain 244 and 31.4 mg of cholesterol per 100g of product, respectively (Aristova and Bekhova 1976). Singh and Gupta (1982) have estimated cholesterol content in different species and reported that goat ghee contains higher cholesterol (236 mg/100 g) than cow (230 mg/100 g) and buffalo (196 mg/100 g) ghee. Pantulu *et al.* (1975) reported cholesterol content for buffalo milk fat in the range of 183 to 383 mg/100

g of fat. Prasad and Pandita (1987) reported the cholesterol content of ghee prepared from the milk of Haryana, Sahiwal and Sahiwal X Friesian cows and from Murrah buffaloes as 303, 310, 328 and 240 mg/100 g fat, respectively. These workers also observed that seasons also affected the cholesterol content of ghee with the highest content in winter (301 mg/100g) and lowest in summer (291 mg/100g fat).

5   Factors affecting the plasma cholesterol in dairy products

Milk contains a large number of bioactive compounds, but milk fat has the largest impact on plasma lipids. The lipid pattern in dairy fat is very complicated and more than 400 different fatty acids have been identified. About 70% of dairy fat contains saturated fatty acids (SFAs) of which the majority (45%) are of 12–16 carbon chain length and 2.7% are trans- fatty acids (LindmarkMånsson *et al.,* 2003), and these have the ability to raise plasma cholesterol. Except for the concentration of different types of plasma lipoproteins that can be affected, their size and composition will change in response to different types of dietary fat. For example, it is suggested that larger sizes of lipoproteins are less atherogenic than smaller sizes (Kawakami A and Yoshida M 2005) and some of the fatty acids typically found in milk fat have been associated with less dense LDL particles (Sjogren *et al.,* 2004). Other milk components like proteins, calcium, and lactose have been suggested to affect lipid metabolism directly or indirectly, but the strongest impact on plasma lipids emerges from the intake of milk fat.

5.1 Saturated fatty acids

Palmitic acid is the major SFA in the diet and also in milk fat with a content of about 30%. Palmitic acid raises the HDL cholesterol less than it raises LDL cholesterol (Grundy 1994). Myristic acid represents 11% of the dairy fatty acids and increase total cholesterol as much as palmitic acid, but does not affect total cholesterol: HDL ratio (Fernandez and West 2005). Lauric acid is the most potent fatty acid in raising plasma total cholesterol, but dairy content is only 3.3%. The increase in HDL cholesterol induced by lauric acid is higher than the increase in LDL and thus the total cholesterol: HDL ratio was decreased when lauric acid was used to replace carbohydrates (Mensink 2003). Stearic acid represents 12% of the dairy fatty acids and improves the plasma cholesterol profile by decreasing total/HDL cholesterol ratio compared to other SFAs. But compared to polyunsaturated fatty acids (PUFA), stearic acid increases LDL and decreases HDL and increase total/ HDL ratio. (Hunter 2003). Other SFAs are short and medium chain length and are mainly considered to be cholesterol neutral(Hayes and Khosla 1992; Lecerf 2009). In a recent meta analysis of prospective epidemiological studies, intake of SFA and risk of coronary vascular disease (CVD) was studied (SiriTarino *et al.* 2010).

5.2 Unsaturated fatty acids

Unsaturated fatty acids are a minor component of milk fat. Oleic acid is considered favorable since it has a cholesterol and triglyceride (TG) lowering effect compared with SFA,

and meals containing olive oil increase the size of postprandial TG rich lipoproteins more than meals with butter (PerezMartinezP *et al.* 2009), which is considered to be less atherogenic.

5.3 Trans fatty acids

Of the seasonal variation of fat in bovine milk, trans fatty acids (*t*FA) have the largest variation and their concentrations are more than twice as high in summer milk as in winter milk (Heck *et al.* 2009). Milk fat is the major source of natural conjugated linoleic acid (CLA) and the predominant isoform is *cis* 9, *trans* 11 CLA, which accounts for 85–90%. The minor *trans*10, *cis*12 CLA isomer has been shown to have a more detrimental effect on plasma cholesterol than *cis* 9, *trans* 11 CLA since it increases total/HDL and LDL/HDL ratios. The effect of natural *t*FA, such as CLA and *trans* 18:1, found in dairy. MotardBelanger *et al.* (2008) suggested that ruminant *t*FA have little impact on cholesterol homeostasis. In this 4 week randomized, crossover controlled study on 38 healthy men, they found that a moderate intake of ruminant *t*FA had neutral effects on plasma lipids, whereas high amounts of both ruminant and industrial *t*FA increased both the total plasma cholesterol by 3 and 2%, respectively, and LDL cholesterol by 6 and 4.6%. In a quantitative review of 39 carefully selected original articles, only including randomized controlled trials in a parallel or crossover design, the authors conclude that all *t*FA increases the LDL/HDL ratio in a linear fashion (Brouwer *et al.* 2010). The effect of ruminant *t*FA and CLA on the LDL/HDL ratio was less than that of industrial *t*FA. In the TRANSFACT study, a randomized controlled trial, 46 healthy subjects consumed 11–12 g/day of either industrial or ruminant *t*FA (Chardigny *et al.* 2008). The major finding was that the natural sources of *t*FA significantly increases HDL and LDL cholesterol concentrations in women but not in men, and the HDL lowering effect is mainly associated with industrial *t*FA. The differences in cholesterol concentrations observed in women were also associated with the size of lipoproteins and *t*FA from natural sources increases the proportion of larger particles. Thus, reducing the intake of ruminant fat will decrease the plasma cholesterol concentration and an improvement of the LDL/HDL ratio is likely. The mechanisms underlying the gender effects and type of isoforms need further investigation.

5.4 Medium chain fatty acids

Medium chain fatty acids (MCFA) are caproic acid (hexanoic acid, $C_{6:0}$), caprylic acid (octanoic acid, C8:0), and capric acid (decanoic acid, $C_{10:0}$). MCFA are present at about 6.8, 6.9, 6.6, and 7.3% (of total fatty acid) in butter, milk, yogurt, and cheese, respectively (Nagao and Yanagita 2010)). MCFA are rapidly hydrolyzed in the gastrointestinal tract (GI tract) and are directly transported to the liver and into the mitochondria of the hepatocytes for oxidation (Aoyama 2007) but some, especially decanoic acid (C10:0) will be incorporated to chylomicron TG. Several studies report effects such as increased lipid oxidation, decreased body weight, increased thermogenesis and energy expenditure from consumption of MCFA and a few studies report a lowering of total cholesterol, LDL, and an increase of LDL particle size (Bourque *et al.* 2003 ; Liu *et al.* 2009). Whether the long term effects on plasma cholesterol levels and an improvement of LDL: HDL ratios are caused by weight reduction or are an actual effect of the MCFA itself is not fully clarified. In a randomized controlled intervention study on

17 healthy individuals, a high intake of 70 g of MCFA per day for 3 weeks was, however, detrimental to plasma lipids with a 12% increase both in LDL cholesterol and LDL/HDL ratio (Tholstrup *et al.* 2004).

5.5 Proteins

In the 1990s, it was suggested that whey proteins had more hypocholesterolemic effect than casein and soy proteins in rats (Nagaoka *et al.* 1992). A decade later, four peptides formed from tryptic cleavage of bovine β-lactoglobulin: IIAEK, GLDIQK, ALPMH, and VYVEELKPTPEGDLEILLQK were found to inhibit cholesterol absorption by increasing fecal output of steroid. Of the four peptides, IIAEK had a higher effect than β-sitosterol. In a recent in vitro study on a human intestinal cell line (NCIH716), the effects both of the specific amino acids leucine, isoleucine and valine, and of whey, skim milk and casein on the expression of lipid-regulating genes were examined (Chen and Reimer 2009). It was found that isoleucine, leucine, valine, and whey down-regulated NiemannPick C1like 1 (NPC1L1), a protein carrier with a critical role in intestinal cholesterol absorption. They conclude that dairy products such as whey, with a high content of branched-chain amino acids can have an effect on cholesterol absorption and possibly also on the plasma cholesterol concentration. The hypocholesterolemic effect of whey has now been confirmed also in humans (Pal *et al.* 2010).

5.6 Lactose

In older human studies, ingesting 80 g of lactose per day as whey resulted in a decreased serum cholesterol (Stahelin and Ritzel 1979). Furthermore, 50 g/day of added lactose to patients with CVD significantly reduced serum cholesterol over a 3 week period (Agarwal *et al.* 1980).

5.7 Fermented dairy products

The effects of fermented dairy products have been studied since the 1970s. The first studies showed that unpasteurized yoghurt decreased serum cholesterol by 5–9%. Later, results from some studies showed no effects of fermented products, but this was explained by the fact that the subjects in the study had a baseline cholesterol concentration of <5.0 mmol/L (Ebringer *et al.* 2008). In a recent study on 34 women, an intake of 125 g of fermented milk three times a day for 4 weeks decreased plasma LDL cholesterol by 12.5–16%. There was also a significant decrease of HDL cholesterol of 10–12% (Andrade and Borges 2009). It is concluded that many strains of fermentation bacteria, but not all are viable enough to reach the lower part of the gut and exert effects on the microbiota, thereby increasing the amount of propionate that has a cholesterol lowering effect. The second effect by which bacteria may influence cholesterol level is by hydrolyzing glycine and taurin conjugated bile acids. By deconjugating bile acids, the excretion of bile acids in feces is increased, which promote the use of cholesterol for synthesis of new bile acids. The use of fermented milk instead of normal milk may therefore be a method to reduce or maintain the plasma cholesterol levels, but the effects on LDL/HDL ratio should be further investigated (Nestel 2005; StOnge *et al.* 2000).

6   Methods for removal of cholesterol

There are various methods and /or processes available in the literature for removal of cholesterol content in butter oil and foods in general and they generally categories into physical, chemical, biological and complexation processes. However, these processes are costly.

6.1 Physical processes

6.1.1 Vacuum steam distillation

In this process, steam is bubbled through liquid milk fat under vacuum. The Omega Source Corporation has acquired this proprietary technology and is currently producing 109.1 kg/day of decholesterolized anhydrous milk fat (Boudreau and Arul 1993). The process reduces free cholesterol by 93 per cent.

Firma Hoche (Speikem, Germany) is also utilizing vacuum stem distillation technology on a commercial scale. The milk fat was reported to be almost neutral in smell and taste with 75 per cent less cholesterol content (Boudreau and Arul 1993).

6.1.2 Short path molecular distillation

Short-path molecular distillation is based on the transfer of molecules from the hot surface of an evaporating liquid to the cooled surface of a condenser through a short path. Lanzani et al. (1994) investigated a new short-path molecular distillation system for the reduction of cholesterol in butter fat and lard. This method however does not remove the high boiling cholesterol ester.

6.1.3 Supercritical fluid extraction

Bradley (1990) have shown that it was technically feasible to produce a milk fat product (80 per cent plastic cream) for a reasonable cost, with at least 90 per cent of the cholesterol extracted using Supercritical carbon dioxide. The finished milk fat retained its color and flavors. In the same study, he described a batch process that removed 90 per cent of the cholesterol from butter by this technology and subsequently yielded a soft spread product (Bradley 1990).

6.2 Chemical process

Gu *et al.* (1994) described Chemical method based on the reaction between cyclic anhydride and the hydroxyl group of cholesterol. The reaction forms monoesters with acryl chains having a terminal acid group. This process could effectively and economically remove up to 40 per cent of the cholesterol from animal fats.

Wrezel *et al.* (1992) were issued a patent "Method for Removing Cholesterol from Edible Oils" based on a procedure that was similar to that of Gu *et al.* (1994).

6.3 Biological process

6.3.1 Cholesterol reductase

Cholesterol reductase is an enzyme that converts cholesterol to coprostanol which is poorly absorbed by humans. Dehal et al. (1991) investigated several cholesterol reductases produced by bacteria or by extraction from leaves of several green plants. They found that cholesterol reductase could convert cholesterol to coprostanol in milk, cream, ground beef and pork. They concluded that these reductases from bacterial or plant sources could be used to decrease the cholesterol content of foods.

6.3.2 Cholesterol oxidases

Several workers (Christodoulou et al. 1994) have reported that cholesterol in foods could be nearly eliminated by cholesterol-degrading enzymes produced by some bacterial strains. These enzymes have been used by Xiansheng et al. (1990).

6.4 Complexation Process

6.4.1 Saponins

Saponins from plants can also be used to selectively bind and precipitate cholesterol. Riccomini et al. (1990) reported an 80% cholesterol reduction in cream by simply mixing cream and saponins for 1 hour at 65 °C, filtering the mixture through Celite and washing with water. The same treatment on anhydrous butterfat produced a 90%t reduction in cholesterol. No adverse flavors could be detected in the final products. Micich et al. (1992) investigated polymer-supported saponins to improve the removal of cholesterol from butter oil. This new support was reusable and reduced the residual saponin in the butter oil. The 1 g polymer-supported saponins can bind 3 mg pure cholesterol in hexane. It was also shown that cholesterol removal from butter oil (dissolved in hexane) was 10-40% less efficient than with pure cholesterol.

6.4.2 β-cyclodextrin

Several studies have been reported on the removal of cholesterol from animal fats with β-cyclodextrin (Davidson 1990; Ockenfull et al. 1991). Yen and Tsai (1995) studied cholesterol removal from a lard-water mixture with β-cyclodextrin, founded that about 90 per cent of cholesterol could be removed from lard.

7   Formation of COPs

Cholesterol present in animal origin foods undergoes autoxidation during processing as well as during storage yielding toxic products. Cholesterol oxidation products (COP) are similar to cholesterol, which contain an additional functional group, such as a hydroxyl, ketone or an epoxide group in the sterol nucleus and/or on the side chain of the molecule. Oxidation of

lipids and sterol (cholesterol) follows the same oxidation patterns such as autoxidation, photo-oxidation and enzymic oxidation, producing relevant hydro peroxides. It is believed that the hydroperoxides derive from oxidation of unsaturated fatty acids play a significant role to facilitate cholesterol oxidation at Δ-5 double bond, which is more sensitive to oxidation (Lercker et al. 2002).

7.1 Autoxidation of cholesterol

The main reaction involved in auto oxidation of cholesterol is a self-catalytic reaction with molecular oxygen Cholesterol autoxidation usually starts at C-7 by abstraction of hydrogen following the addition of an oxygen molecule forming primary COP, isomers of 7-hydroperoxycholesterol. The 7-hydroperoxycholesterols can further convert into 7α-hydroxycholesterol and 7β-hydroxycholesterol, which are commonly found in food (Lercker et al. 2002). 7-ketocholesterol is formed by dehydration of isomeric 7-hydroxycholesterol in the presence of radicals. It is a major COP in the food matrix. Formation of isomeric epoxy cholesterols occurs due to the interaction between cholesterol and hydroxyl radicals and these epoxy compounds can be hydrolyzed in acidic medium converting them into most toxic triols. The side chain oxidation occurs at C- 20 and C-25 positions resulting in the production of relevant hydroperoxide, 20-hydroperoxide and 25-hydroperoxide respectively, which can further reduce into 20α -hydroxycholesterol and 25-hydroxy cholesterol (Lercker et al. 2002).

7.2 Photo oxidation of cholesterol

In photooxidation of cholesterol, singlet oxygen is formed from triplet oxygen by light in the presence of an active sensitizer (natural pigment or synthetic colorant). Cholesterol can react with singlet oxygen in the presence of photo-sensitizer, forming dominant hydroperoxide at C-5. A part of this hydroperoxide converts into 5α-hydroxycholesterol and the other part is further converted into stable 7-hydroxiperoxide and 6-hydroperoxide that are present in minor amounts. 7-hydroperoxides can be converted into isomeric 7-hydroxycholesterol and into 7-ketocholesterol at the same time 5-hydroxycholessterol can be formed (Lercker 2002).

7.3 Enzymic-oxidation of cholesterol

Some enzymes in food oxidize cholesterol. Available reports show that the conversion of 5α- hydroperoxides (5α-HPC) into 7α-HPC, epimerization of 7α-HPC into 7β-HPC, and formation of 7-HC epimers from the corresponding hydroperoxides occur by enzymatic reactions. But this has to be studied to a further extent due to the fact that these products can be formed by the usual non-enzymatic reactions (Lercker et al. 2002). The main enzymes involved in the enzymatic oxidation of cholesterol are Monooxygenase, dioxygenase, dehydrogenase, and oxidases are. The COPs like 7α hydroxycholesterol, 25-hydroxycholesterol, 20α-hydroxycholesterol, (25R)-26-hydroxycholesterol, 22Rhydroxycholesterol, are produced by enzymatic oxidation of cholesterol (Lercker et al. 2002).

These oxides are produced endogenously in human tissues during conversion of cholesterol to bile acids and steroid hormones and they can oxidize cholesterol effectively in the presence of human gastric fluid and it acts as a better medium for cholesterol oxidation and produce COP in vivo (Dobarganes et al. 2003). These enzymic and non-enzymic reactions can occur separately or/and simultaneously in food production, processing, distribution and storage (Ubhayasekera et al. 2004; Dobarganes and Marquez-Ruiz 2003).

Cholesterol oxidation products (COP) are similar to cholesterol but they contain an additional functional group like hydroxyl, ketone or an epoxide group in the sterol nucleus and/or on the side chain of the molecule. The COPs can be formed by mainly 3 major oxidation pathway that is, autoxidation, photo-oxidation and enzymatic oxidation of cholesterol. The main reaction involved in autooxidation of cholesterol is a self-catalytic reaction with molecular oxygen. Cholesterol autoxidation usually starts at C-7 by abstraction of hydrogen following the addition of an oxygen molecule. This will form primary COP, isomers of 7-hydroperoxycholesterol. The 7-hydroperoxycholesterols can further convert into 7α-hydroxycholesterol and 7β-hydroxycholesterol. These 7α-hydroxycholesterol and 7β-hydroxycholesterol are commonly found in food (Lercker and Rodriguez-Estrada 2002).

7.4 COPs in fresh milk and dairy products

Formation of COP in fresh milk or fresh dairy products is very low due to the low level of oxygen. Bican (1984) studied COPs content in raw milk using HPLC but he did not find any detectable COPs in fresh milk. Kou and Holmes in 1995 studied COPs in fresh cream as well as fresh butter, but they did not find any detectable COPs.

7.5 COPs in heat-treated dairy products

Milk was heated under different time-temperature conditions, varying from pasteurization to UHT-treatment but showed no (Cleveland and Harris 1987) COP formation. COPs were detected in butter after different heat treatments. The higher the temperature used and the longer the storage time, the total COPs were produced in more amount(Pie et al. 1990).Unsalted butter oil contained 300 mg/kg ketocholesterol and 200 mg/kg a-epoxycholesterol which were two to three times higher than in salted butter oil (Sander et al. 1989b).

7.6 COPs in heat-treated and/or stored dairy products

The spray dried skim milk powders were analyzed after storage of 13–37 months contained substantial amounts of total COPs. They contained substantial amounts of total COPs (between 20 mg/kg and 78 mg/kg in total lipids; (Nourooz-Zadeh and Appelqvist 1988a). Milk powder packaged in PE pouches had a total COPs content of 15.6% of the original cholesterol content. Samples packaged in glass vials with and without oxygen absorbers had COPs contents of 0.7% and 5.9% of the original cholesterol, respectively.

7.7 COPs in dairy products after light exposure

Cholesterol in butter was oxidized during exposure to fluorescent light. The COPs were more concentrated at the surface than throughout the entire butter block (Luby et al. 1986b). The 7-ketocholesterol was seen in Feta cheese exposed to fluorescent light at 41°C /30 days compared to day 0 (Nielsen et al. 1995).

8 Significance of COPs on health

8.1 Atherosclerosis and inflammation

Atherosclerosis is the most well-known condition that involves oxysterols. Atherosclerosis means the accumulation of cholesterol deposits in macrophages in the walls of large and medium arteries. Subsequent formation of atherosclerotic plaque gradually narrows vessel lumen, leads to thrombosis, and compromises oxygen supply to target organs, eventually causing heart attack and stroke-the major sources of mortality in the developed world. Cholesterol present in atherosclerotic lesions comes mostly from low density lipoproteins. One of the popular hypotheses explaining atherogenesis (Chisolm and Steinberg, 2000) states that it is the oxidation of LDL that triggers its internalization by macrophages through the so-called scavenger receptor pathway (Goldstein et al. 1979). Significant amounts of 27-OH-chol, 7-Keto-chol, and 7a/b-OH-chol are often found in lesion and foam cells, where their levels are at least two orders of magnitude higher than in plasma (Brown and Jessup 1999). These oxysterols constitute 75–85% of all oxysterols in plaque and are present mostly (>80%) as mono- and diesters. Many other oxysterols, derived from diet, in vivo oxidation, or enzymatic reactions during cholesterol catabolism have been detected and are claimed to play an active role in plaque development.

8.2 Neurodegenerative diseases

Neurodegenerative diseases, including Alzheimer's, Parkin-son's, Huntington's, and multiple sclerosis, constitute another set of conditions that are often related to oxysterols. The human brain, representing only 2% of total body weight, contains 25% of total body cholesterol, mainly in unesterified form, distributed between myelin (70%), glial cells (20%), and neurons (10%) (Maxfield and Tabas 2005). Since the brain is isolated by the blood–brain barrier, its cholesterol has to be synthesized in situ, and its excess is eliminated after conversion to 24-OH-chol by 24-hydroxylase (CYP46A1) (Bjorkhem 2006). In contrast to cholesterol, this chain-oxidized sterol can easily pass through the blood brain barrier and can be metabolized by the liver. Also, 27-OH-chol, the most abundant oxysterol in human circulation, and 7a-hydroxy-3-oxo-4-cholestenoic acid were shown to pass the blood brain barrier (Bjorkhem et al. 2009). In this context it is not surprising that oxysterols play an active role in pathologic conditions related to the deregulation of brain cholesterol homeostasis (Martins et al. 2009).

In the pathogenesis of AD, oxysterols were found to be involved in the alteration of cholesterol metabolism, modulation of neuroinflammation, aggregation and accumulation, and neuron death (Gamba et al. 2015). Parkinson's disease (PD) is the most common movement disorder. It is characterized by the aggregation of a-synuclein protein in Lewy body inclusions and loss of dopaminergic neurons in the substantia nigra (Rantham Prabhakara et al. 2008). Oxysterols (mainly 24S-OH-chol, 27-OH-chol, and secosterol) are particularly responsible for causing a-synuclein aggregation and destruction of dopamine-containing neurons (Bosco et al. 2006). Also, the levels of oxidative cholesterol metabolites are higher in the cerebral cortex of PD patients. The level of 24S-OH-chol in cerebrospinal fluid, but not in blood, correlates with the duration of the disease (Bjorkhem et al. 2013.

The antiviral properties of 25-OH-chol were first identified for enveloped viruses (that is, vesicular stomatitis virus, human immunodeficiency virus (Liu et al. 2011b)) and later for nonenveloped ones (that is, human papillomavirus-16, human rotavirus, and human rhinovirus) (Civra et al. 2014). Recently, its antiviral action against hepatitis C virus has also been documented (Anggakusuma et al., 2015; Xiang et al., 2015). Since both 25-OH-chol and statins have similar antiviral effects and mutual targets, it has been proposed that their synergy could be used in therapy (Chen and Peng 2015).

8.3 Cancer

Oxysterols are associated with cancers of the colon, lung, skin, breast, prostate, and bile ducts. Procarcinogenic oxysterols might exert their effect at three stages of carcinogenesis: by induction of DNA damage, by induction of cyclooxygenase-2 expression, or by stimulation of tumor cell migration (Zarrouk et al. 2014). In vitro, oxysterols were shown to interfere with proliferation and cause the death of many cancer cell types, while having little effect on senescent cells (de Weille et al. 2013). Certain oxysterols can exhibit anticancer effects, possibly via modulation of cholesterol efflux, protein kinase B, or LXRs. For example, treatment with 22R-OH-chol, 24S-OH-chol, 7a-OH-chol, 7b-OH-chol, 25-OH-chol, 5a, 6b-Epoxy-chol, and 3b, 5a, 6b-3OH-chol suppresses the proliferation of human prostate, breast, colon, lung, and leukemia cancer cells (Lin et al. 2013).

8.4 Eye diseases

In age-related macular degeneration (AMD), cataracts, and opacified corneas, the involvement of oxysterols is also widely suspected (Vejux and Lizard 2009). The accumulation of 7-Keto-chol in lipoprotein deposits in the choriocapillaris, Bruch's membrane, and retinal pigment epithelium (RPE) can have cytotoxic, pro-oxidant, and pro-inflammatory activities (Rodriguez and Larrayoz 2010). All those processes can contribute to AMD, causing irreversible central vision loss and blindness. It seems that, to some extent, 7-Keto-chol can be detoxified locally by enzymatic 25R, 26-hydroxylation in RPE (Javitt 2008). 27-OH-chol was shown to cause Ab accumulation and oxidative cell damage in RPE (Dasari et al. 2010), linking the pathogeneses of AMD and AD. During aging, oxysterols (that is, 7b-OH-chol, 7-Keto-chol, 5a, 6a-Epoxy-chol, 20a-OH-chol, and 25- OH-chol) were shown to accumulate in human lenses, participating in clouding of the lens (cataracts), the dominant form of blindness (Vejux and

Lizard 2009). Cataracts are associated with smoking, diabetes, glucocorticoid therapy, and excessive exposure to sunlight, all of which are known to induce oxidative stress (Zarrouk et al. 2014). An opacified cornea is also characterized by the accumulation of various oxysterols (that is, cholesta-3, 5-dien-7- one, cholest-4-en-3-one) (Vejux and Lizard 2009).

8.5 Other conditions

The role of oxysterols has also been postulated in type 2 diabetes (Sottero et al. 2015), sensorineural hearing loss (Mal-grange et al. 2015), and chronic inflammatory processes of the gallbladder, anorexia, and chronic renal failure (Sottero et al. 2009). Intoxication with ethanol was found to increase the levels of 7-Keto-chol, 7a-OH-chol, and 7b-OH-chol in the liver, skeletal muscle, jejunum, and heart of rats (Sottero et al. 2009). Estrogen signaling is critical for maintaining proper bone density. Increased levels of 27-OH-chol were shown to decrease bone mineral density. These data provide evidence for interactions between estrogen signaling, cholesterol and metabolic disease, and osteoporosis (DuSell et al. 2010).

9   Methods for estimation of cholesterol and COPs

A number of methods are available in the literature and many of these are being tried in clinical and food laboratories. Most of these methods for cholesterol estimation were initially developed for blood plasma and then applied to foods. Various analytical approaches employed for cholesterol estimation are (1) gravimetric methods, (2) colorimetric methods (for example, Liebermann-Burchard reaction, o-Phthaldehyde and ferric chloride method),(3) chromatographic methods (GLC, HPLC), (4) Infrared based methods (FT-NIR, - MIR), and (5) Enzymatic method(Makwana 2007).

The quantification of COP in food is difficult because there are interruptions by large amounts of interfering cholesterol, triglycerides, phospholipids and other lipids. On the other hand technical difficulties associated with cholesterol oxide analysis play a major part due to similar chemical structures and presence of cholesterol oxides at trace levels. Therefore, the extraction, purification, and detection methods that use to identify and quantify these products play a major role (Ulberth and Buchgraber 2002; Guardiola et al. 2004). COPs are fat-soluble compounds. Many solvents or solvent combinations can be used to extract COP and the most important factor to be considered at this point is the complete recovery. The various combinations of the solvents are chloroform/methanol; n-hexane/2-propanol; dichloromethane/methanol and so forth. HPLC is a better method to analyze COP due to the possibility of separation, detection and quantification, especially the thermo-labile molecules (for example, cholesterol hydro peroxides). The GC is the common method of COP determination using deivatized COP as trimethylsilyl ether (TMSE) derivatives (Ubhayasekera et al. 2004).

10 Methods for estimation of cholesterol

Increasing awareness about health consciousness among consumers, formulation of mandatory guide lines which include the declaring of nutritional facts on the label of food packet have stressed much emphasis on estimation of cholesterol in food products. A number of methods are available in the literature and many of these are being tried in clinical and food laboratories. Most of these methods for cholesterol estimation were initially developed for blood plasma and then applied to foods. Various analytical approaches employed for cholesterol estimation are listed bellow and discussed in subsequent sections.

10.1 Gravimetric Methods

Windous (1910) presented a reaction between alcoholic solution of cholesterol and alcoholic solution of digitonin ($C_{56}H_{92}O_{29}$) which results in precipitation of 1:1 molecular complex of cholesterol and digitonin as cholesterol digitonoids.

$$C_{56}H_{92}O_{29} + C_{27}H_{46}O \rightarrow C_{56}H_{92}O_{29} + C_{27}H_{46}O$$
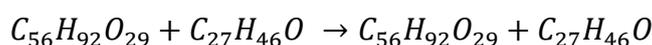
This reaction became a platform for several gravimetric methods of estimating cholesterol in fats and oils. Bloor (1916) observed that saponification was not necessary in the above method, as cholesterol in both forms gives colour. He suggested a method for extraction of cholesterol but the results obtained by his method gave much higher result than the Windous method (1910).

10.2 Colorimetric Methods

10.2.1 Liebermann-Burchard (LB) reaction method

Ghee (0.2 to 0.25) g of ghee dissolved in chloroform (3 ml), 4 ml L.B. reagent (acetic anhydride: H2SO4 :: 20:1, freshly prepared at 0 °C) was added and the mixture incubated at 25 °C for 12 min. The optical density was recorded at 650 nm in the colorimeter, taking care that the measurements were completed within 15 min after the addition of LB Reagent. The amount of cholesterol was calculated by plotting a standard curve, prepared under same conditions simultaneously. They compared their results with that of gravimetric method of Windaus (1910) and found that the method gave the comparable result with gravimetric method. Cholesterol content in cow and buffalo ghee was found to be 310 and 270 mg per 100 g of ghee, respectively (Bindal and Jain, 1973)

Liebermann-Burchard test is used for the colorimetric test to detect cholesterol, which gives a deep green color. The formation of a green or green-blue color after a few minutes mean is the positive result. This color begins as a purplish, pink color and progresses through to a light green then very dark green color. The color is due to the hydroxyl group (-OH) of cholesterol reacting with the reagents and increasing the conjugation of the unsaturation in the adjacent fused ring. In this reaction, the acetic acid in the Liebermann reagent reacts with cholesterol in the sample, which gives a green color whose absorbance, can be determined by UV-visible spectrophotometer at 640 nm (Burke et al. 1974).

10.2.2 o-Phthaldehyde method

Bachman et al. (1976) used o-phthaldehyde method for determination of cholesterol in dairy products. Accurately weighed dairy products were saponified and the unsaponifiable matter was extracted using 10 ml of hexane. An aliquot (4ml) of hexane extract was evaporated and 4 ml of o-phthaldehyde reagent (50 mg/100 ml in glacial acetic acid) was added and mixed properly. After 10 min, 2 ml of concentrated H_2SO_4 was added and absorbance was taken after 10 min at 550 nm. These results were compared with GLC method for cholesterol estimation and found good agreement between the two methods. Using this method, they reported 13.2, 46.1, 104.1, 95.3, 97.4, 78.4 and 24.2 mg of cholesterol per 100 g of sample in milk, vanilla ice-cream, cheddar, swiss, processed cheese and Creamed Cottage Cheese, respectively. Recovery of the method was found to be 99.79%.

10.3 Chromatographic Methods

10.3.1 Gas-liquid chromatographic methods

Cholesterol determination by gas-liquid chromatography (GLC) is usually more accurate than colorimetric procedures (Vanzetti 1964). Numerous studies have been done on GLC method of cholesterol determination, but in all cases, a trimethylsilyl (TMS) ether derivatization of sterols is involved which is time-consuming. In the past, most studies focused on a quick, accurate and simple method to analyze cholesterol. For example, gas chromatography (GC) with capillary columns, in many cases, a method for routine analysis of sterols, especially for cholesterol (Itoh et al. 1973).

Punwar (1975) developed a widely used approach for determining cholesterol in foods. The method involved lipid extraction, saponification and silyl derivatization followed by estimation through gas chromatography. However, the lipid extraction and saponification steps in this method were cumbersome, the derivatizing reagents were unstable and cholesterol was thermally decomposed in the GC column (Kovacs et al. 1979).

To overcome these problems, Kovacs et al. (1979) proposed a method in which derivatization step of cholesterol was removed. They used a gas chromatograph equipped with flame ionization detector (FID) and glass column packed with Gas Chrom Q and coated with 3% OV-17. The column was operated at 230°C using helium as carrier gas at a flow rate of 40 ml/min. Injector and detector temperatures were 235 °C and 240 °C respectively. For quantification purpose, 5α-cholestane was used as the internal standard and all sterols were identified by comparing their retention times with authentic standards. Prior to GLC analysis, sterols were saponified and extracted into hexane.

Tsui (1989) developed a method for rapid determination of total cholesterol in homogenized milk. The milk sample was saponified in ethanolic KOH in the presence of an internal standard, 5α-cholestane. Cholesterol and cholestane were then isolated by solid phase extraction on a non-polar absorbent and eluted with the organic solvent. The evaporated extract was derivatized and analyzed by capillary gas chromatography. The average recovery of cholesterol added to milk prior to saponification was 95% and the average relative standard deviation for repeated analyses was 2%. The limit of detection for this method was 2 mg per 100 g.

Prior to GC analysis, cholesterol was saponified and extracted from the food sample (containing about 1 mg of cholesterol). Unsaponifiable matter was extracted using n-hexane and TMS ether derivatization of cholesterol was performed and dissolved in 1 ml hexane. 2 µl hexane solution was injected into GC and GLC was performed using the following analytical condition: injection port and detector block- 330 °C, column oven temperature program -250-320 °C at 4 °C/min, column inlet helium pressure- 1.75 kg/cm2 at 250 °C and split ratio- 60:1. Internal standard (5α-cholestane) and TMS ether derivative of cholesterol eluted at 16 and 20 min respectively, their peak area was determined and concentration of cholesterol was calculated.

Standard method of AOAC (2005) for estimating cholesterol in foods involves saponification, extraction, derivatization, and GC analysis. In the method, 2-3 g of sample was saponified with 40 ml of 95% ethanol and 8 ml of 50% KOH solution by heating on water bath for 70 ± 10 min. Unsaponifiable matter was extracted into 100 ml toluene and it was washed with 110 ml of 1 M KOH, 40 ml 0.5 M KOH, and 40 ml water (washing with water was carried out for 3 times), respectively. Then the toluene layer was passed through Na2SO4 and the solvent layer was allowed to stand for 15 min. The sterols present in the solvent layer were derivatized to trimethylsilyl esters (TMS) using dimethylformamide(DMF), hexamethyldisilane (HMDS), and trimethylchlorsilane (TMCS). For quantification purpose, 5α-Cholestane was used as internal standard. In GC analysis, 1 µl of test portions was injected into capillary column and hydrogen flame ionized detector was employed to detect the sterol peaks. The operating conditions maintained were; injector temperature 250 °C, detector temperature 300 °C, and column temperature 190 °C.

10.4 High performance liquid chromatographic (HPLC) methods

High-performance liquid chromatography (HPLC) techniques involve the use of nonaqueous reversed phase systems with saponified or esterified derivatives to determine cholesterol in foods (Hurst et al. 1983). The mobile phases in these reversed phase systems range from nonpolar that is, 2 propanol/hexane (Newkirk and sheppard, 1981) to extremely polar that is, methanol (Sugino et al. 1986). Detection of cholesterol in extracted lipids by HPLC is usually based on absorbance of short- wavelengths UV light (200-210 nm; Duncan et al. 1979; Carrol and Rudel 1981). Because of their simplicity and wide area of application, several liquid chromatographic methods have been developed for cholesterol determination coupling with UV-absorbing derivatives (Newkirk and Sheppard 1981).

Oh et al. (2001) developed a sensitive high-performance liquid chromatographic method to determine the cholesterol content in milk and milk products. They compared various extraction process and various mobile phases for separating cholesterol by HPLC in order to develop a simple and accurate method. They observed that solid phase extraction (SPE) had a high recovery with shorter extraction time and the process was highly reproducible in comparison to liquid-liquid extraction. Further, acetonitrile: methanol: 2-propanol was found to be superior to the other mobile phase systems for separating cholesterol. HPLC was performed using a multi-solvent delivery pump equipped with a 20 µl injection loop and a C18 column. Maintaining the flow rate of mobile phase at 1.6 ml/min, elution of cholesterol was

monitored at 205 nm using UV detector method, again, accurately predicted the cholesterol content in a variety of dairy products.

10.4.1 Methods Based On Infra-Red Spectroscopy

Paradkar and Irudayaraj (2002a, b) developed two methods based on principle of FT-IR and FT-NIR spectroscopy for determination of cholesterol in dairy products.

In the FT-IR method, 1.5-2.5 g of sample was mixed with 9 ml of ethanol and 1 ml of potassium hydroxide solution (50 g per 100 ml) and vortex mixed for 20 sec. The capped tube was then placed in a water bath maintained at 60 °C for 1 hr in order to have complete saponification and stirred continuously at 200 rpm. After cooling to room temperature, 5 ml of deionized water and 10 ml of hexane were added and vortexed for approximately 2 min. The sample was then centrifuged for 3 min at 2000 rpm and the hexane layer was transferred into a clean tube. Another 10 ml of hexane was added to the aqueous phase. The extraction and centrifugation steps were repeated. The combined hexane extract was then used for cholesterol determination. The hexane extract was evaporated to dryness and the sample was re-dissolved in 5 mL of chloroform and used for FTIR analysis. They concluded that FTIR (mid-infrared) spectroscopy was successfully used for the rapid estimation of cholesterol in commercial dairy products such as milk, milk powder, yogurt, cheese, grated cheese, and butter. The Partial Least Square (PLS) calibration model in the region between 2800 cm–1 and 3200 cm–1 was found to be the most accurate ($R2 = 0.99$). The method developed was fast and less expensive than the conventional method and could be accomplished in less than 5 min.

10.4.2 Electrophoretic Method

Xu et al. (2002) described a simple non-aqueous capillary electrophoresis (NACE) method for the rapid quantification of cholesterol in egg yolk and milk. The samples were subjected to saponification and then quantified by NACE, in which 100 mM sodium acetate-acetic acid in methanol was employed as the running buffer. The correlation coefficient between the cholesterol concentration and the corresponding peak area was 0.999. The detection limit of cholesterol was 5 g/ml (twice the signal-to-noise ratio). They recommended this method as a routine method for the rapid and sensitive determination of cholesterol in foods.

10.4.3 Enzymatic Method

Enzyme kits containing peroxidase, cholesterol esterase, and cholesterol oxidase have been employed satisfactorily to determine cholesterol in egg yolk (Jiang et al. 1990; Van Elswyk et al. 1991).

11 Analysis of cholesterol oxidation products

The COP levels reported for similar food, sometimes not lie in the same range and sometimes a considerable difference could be observed. These differences are due to the differences in manufacturing technology and analytical procedure. This brings up a very important need of accurate quantification (McCluskey and Devery 1993; Guardiola et al. 2004). The quantification of COP in food is difficult because there are interruptions by large amounts

of interfering cholesterol, triglycerides, phospholipids and other lipids (Ulberth and Buchgraber 2002). On the other hand technical difficulties associated with cholesterol oxide analysis play a major part due to similar chemical structures and presence of cholesterol oxides at trace levels (parts per million). Therefore, the extraction, purification, and detection methods that use to identify and quantify these products play a major role (Ulberth and Buchgraber 2002).

The analytical procedure should be designed in a way to guarantee efficient recovery of COP from food matrix or biological fluids and minimization of the generation of artifacts during sample clean-up and work-up steps. Most common analytical protocol used for COP analysis, has more or less the same route: (i)Extraction of COP from food matrix or biological system; (ii)Purification of the sample extract; (iii) Derivatization to suitable compounds and (iv)Quantification with a suitable chromatographic method (Ulberth and Buchgraber, 2002; Guardiola et al. 2004)

11.1 Extraction, purification and enrichment

COPs are fat-soluble compounds. It is very difficult to isolate COPs due to low levels from the other fat-soluble substances in food matrices, such as mono, di, tri acylglycerols, esterified and free cholesterol, free fatty acids and phospholipids. Many solvents or solvent combinations can be used to extract COP and the most important factor to be considered at this point is the complete recovery. These preparation steps are very important due to their possible contribution to the formation of artifacts, COP recovery, peak resolution, detection efficiency, identification and quantification (Rodriguez-Estrada and Caboni 2002).

Choose of a solvent or a solvent system play a significant role as the solvent system must be capable of disrupting the associative forces binding the cholesterol and lipids to non-lipid material in biological matrix. Therefore no single solvent is capable of complete cholesterol extraction whereas a mixture of solvents is used (Ulberth and Buchgraber 2002). The most commonly reported methods for cholesterol extraction are:

1. Methods involving a preliminary fat extraction followed by a saponification

    a. Chloroform/methanol (2:1, v/v); (Folch et al. 1957)
    b. n-hexane/2-propanol (3:2, v/v); (Hara and Radin 1978)
    c. Dichloromethane/methanol (9:1, vol/vol); Dry column method (Higley et al. 1986; Zubillaga and Maerker 1991).

2. Methods that directly treat the sample

    a. Direct saponification or trans esterification (Dionisi et al. 1998)

Apart from these methods, reports of usage of single solvents like chloroform and Soxhlet extractions using solvents such as tert-butylmethyl ether and dichloromethane are also available (Ulberth and Buchgraber, 2002). Very few reports are available on the comparison of different extraction methods. Dionisi et al. (1998) have compared commonly used four lipid extraction methods, direct saponification, modified Maxwell's method, modified Folch's method and modified Radin's method by extracting lipid followed by saponification, solid

phase extraction (SPE) column purification and GC-MS (gas chromatography coupled to mass spectrometric detector) quantification.

Direct method, Maxwell's and Folch's methods gave comparable results. But Radin's method had shown drastic low COP recovery, probably due to the apolar solvents usage in fat extraction. Interestingly, Radin's method has shown that spiking gives better recovery (Ulberth and Buchgraber 2002). Accelerated solvent extraction (ASE) is well known for high efficiency in lipid extraction but not many available reports on COP analysis. In a total lipid extract 11 triglycerides and/or phospholipids are major compounds whereas sterols are present in minor levels and COPs are in trace levels. To analyze with less interference and for clear detection these COP need to be enriched. There are two enrichment methods such as saponification and trans esterification (Ulberth and Buchgraber 2002).

Saponification is employed to remove triglycerides, free fatty acids and water-soluble impurities during extraction of COP by converting them to water-soluble derivatives using methanoic or ethanoic NaOH or KOH. COP extraction to a suitable organic solvent is done after adding water to the saponified mixture. The unsaponifiable fraction contains COP. Two saponification procedures, cold and hot saponification are often used. Cold saponification at 25 °C for 18-22 hours has shown high recovery (Tai et al. 1999) and low artifact formation. Use of hot saponification at 60 °C for 45-60 minutes has reduced the saponification time. Hot saponification leads to artifact formation by degrading 7-keto and isomeric epoxides. To overcome this problem purification of COP in silica gel- or C18 cartridges have been used (Ulberth and Buchgraber 2002; Guardiola et al. 2002).

Apart from the disadvantages of possible artifact formation, saponification also has some practical drawbacks. The saponified triglycerides form a soap solution giving bad separation of the evolved emulsions and micelle formation leads to loss of compounds of interest. (Schmarr et al. 1996).The other method used to free the sterol and its oxides from the bulk of the accompanying lipids is trans esterification. The trans esterification is a method with mild conditions converting esterified oxysterols and triglycerides into fatty acid methyl esters. After esterification, the lipid primarily consists of acid methyl ester, free cholesterol and its oxidation products and some minor apolar and polar compounds. This mixture could be separated by a solid phase extraction column and stepwise elution with solvent of increasing polarity. No artifact formation is detected with this method (Schmarr et al. 1996).

Solid phase extraction (SPE) column uses the polarity differences in the matrix component to separate a mixture of compounds with different polarity. Phospholipids, cholesterol and its oxidation products and cholesteryl esters are most, medium and least polar respectively. Therefore, SPE method helps to separate COP with minimum contamination with coeluted cholesterol and phytosterol, if present. The advantages of using a SPE column are as follows: (i) no destruction of sensitive COP as no strong alkali is used (ii) artifact formation is minimized due to the removal of free cholesterol. The factors such as pressure of air, light, peroxides in solvents, heat and contact with reactive groups (for example, silicic acid) leads to artifact formation (Ulberth and Buchgraber 2002). There are several different matrices used in SPE columns whereas the COP analysis has been reported with silica (Si-), Florisil, aminopropyl-modified silica (NH2-) and octadecyl-modified silica (ODS-) (Ulberth and Buchgrabe 2002).

11.2 Thin-layer chromatography (TLC)

TLC is an efficient, rapid and simple technique used to separate, isolate and identify COP in crystalline cholesterol, food and biological samples. Use of TLC is limited to the separation of COP but not for accurate quantification. TLC is capable of separating hydroxycholesterols but not cholesterol hydroperoxides (Lebovics 2002).TLC is useful in confirming the identity of COP based on the color development after spraying sulfuric acid and observation under UV (Tai *et al.* 1999). There are reports of separation of COP such as 7α-HC, 7β-HC, and 5,6-CE with TLC method and purification of COP (Nourooz-zadeh and Appelqvist 1988).

The major drawbacks of TLC analysis are incapability of 5,6-EP isomer separation, tedious method, inaccuracy of quantification of COP based on the spot area, poor resolution, instability, time consuming procedure. There are many advantages of TLC such as parallel analysis of many samples, possibility of fast screening, ability to guess the amount of COP, purification to a certain level and low cost (Lebovics 2002).

11.3 High Performance Liquid Chromatography (HPLC)

COP_S are present in small quantities in food and quantification of them are challenging. The isomers of some COP show similar chemical, spectrometric and fragmentation characteristics. All these reasons stress the requirement of high sensitive methods and systems to identify and quantify COP. The choice of analytical tool is governed by, the type of matrix and the scope of the analysis (Rodriguez- Estrada and Caboni, 2002).

HPLC is a better method to analyze COP due to the possibility of separation, detection and quantification, especially the thermo-labile molecules (for example, cholesterol hydroperoxides) as it is a non-destructive technique. This is a versatile analytical system due to its ability to couple into many detector systems, and to run in normal or reverse phase modes using different column types and dimensions and mobile phase compositions, leading to different separation, identification and quantification. HPLC coupled to MS detectors has become a very powerful tool over the past years due to the increment of the sensitivity (Rodriguez-Estrada and Caboni 2002).

Detectors connected to HPLC can give different results. In an HPLC analysis with a spectrometric detection, it has been reported the inability in detection of some human harmful COP due to poor UV absorption Careri *et al.* 1998).

11.4 Gas Chromatography (GC)

The gas chromatography is the common method of COP determination using derivatized COP as trimethylsilyl ether (TMSE) derivatives. This derivatization method avoids peak tailing and it improves the thermal stability of some hydroxy cholesterols. There are few factors affecting COP response in GC such as injection technique and conditions, reagents, and conditions use to get derivatives (Guardiola, *et al.* 2002).GC gives an accurate quantification of COP with a flame ionization detector (FID), or with a mass selective detector (MS/MSD). It has been observed a clear separation of COP with a good resolution when a silica capillary (dimethyl poly siloxane) column connected to GC (Tai *et al.* 1999). The best combination of

techniques to identify COP rapidly and sensitively is GC-MS with a selected ion monitoring mode (SIM) and a capillary column. The outcome of this, the mass spectrum can be compared with the chemical library to identify COP with high accuracy (Tai *et al.* 1999; Guardiola *et al.* 2002).

12 Conclusion

Cholesterol is one of the components of biological membranes and found in all body tissues. Though cholesterol is vital to the body, the plasma cholesterol is associated with coronary heart disease. Cholesterol undergoes autoxidation, photo oxidation, and enzymatic oxidation, producing relevant hydroperoxides. The COPs are associated with various health diseases. Accurate determination of cholesterol contents in foods is very essential for the regulatory aspects of food labeling, especially because of the fact that cholesterol is related to human health. Various analytical approaches have been described in the literature for example, colorimetric, gravimetric, chromatographic, infrared, and so forth. For estimation of cholesterol and its oxidized products in dairy and food products.

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