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Analysis, Formation and Inhibition of Cholesterol Oxidation Products in Foods: An Overview (Part I)

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ABSTRACT

Cholesterol oxidation products (COPs) formed in cholesterol-containing foods during heating or illumination have been found to impart a potential hazard to health. Numerous studies have indicated that COPs may have several adverse biological effects, such as mutagenicity, carcinogenicity, angiotoxicity, cytotoxicity, atherogenicity, atherosclerosis, cell membrane damage and inhibition of cholesterol biosynthesis. Therefore, the safety of COPs has become a major concern for the public. This paper is an overview of analysis, formation and inhibition of COPs in foods. COPs are routinely extracted by organic solvents, followed by saponification and solid phase extraction for enrichment of COPs, and separation and identification by thin-layer chromatography (TLC), high-performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS). The identification and quantification of COPs using the GC-MS technique was found to be rapid and sensitive, however, the formation of artifacts is a major drawback. The HPLC method failed to resolve several geometrical isomers, and double bond-free COPs such as isomeric 5,6-epoxides and triol could not be detected with UV. The oxidation of cholesterol can be accelerated by heating, pH, storage conditions, the presence of food components and other factors. Several COPs are commonly present in food systems, including 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 7-keto, 20α-OH, 25-OH and triol. Of these COPs, 5,6α-EP, 5,6β-EP, 7-keto, 20α-OH and 25-OH are primary oxidation products, while 7α-OH, 7β-OH and triol are secondary products. Some antioxidants have been found to reduce the formation of COPs in an appropriate concentration. Also, adequate packaging is necessary to provide a physical barrier for air and light, and thus minimize cholesterol oxidation. Further research is necessary to study how to inhibit COPs formation in foods.

Key words: **cholesterol oxidation products, HPLC, GC-MS, processing method.**

INTRODUCTION

Cholesterol is widely distributed in foods of animal origin, and is susceptible to oxidation to form cholesterol oxidation products (COPs) during heating and illumination[1-4]. More than 80

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COPs have been identified so far. The most common COPs present in foodstuff include the following: 7-ketocholesterol (7-keto), 6-ketocholesterol (6-keto), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), 5,6α-epoxycholesterol (5,6α-EP), 5,6β-epoxycholesterol (5,6β-EP), 25-hydroxycholesterol (25-OH), 20-hydroxycholesterol (20-OH) and cholestanetriol (triol).

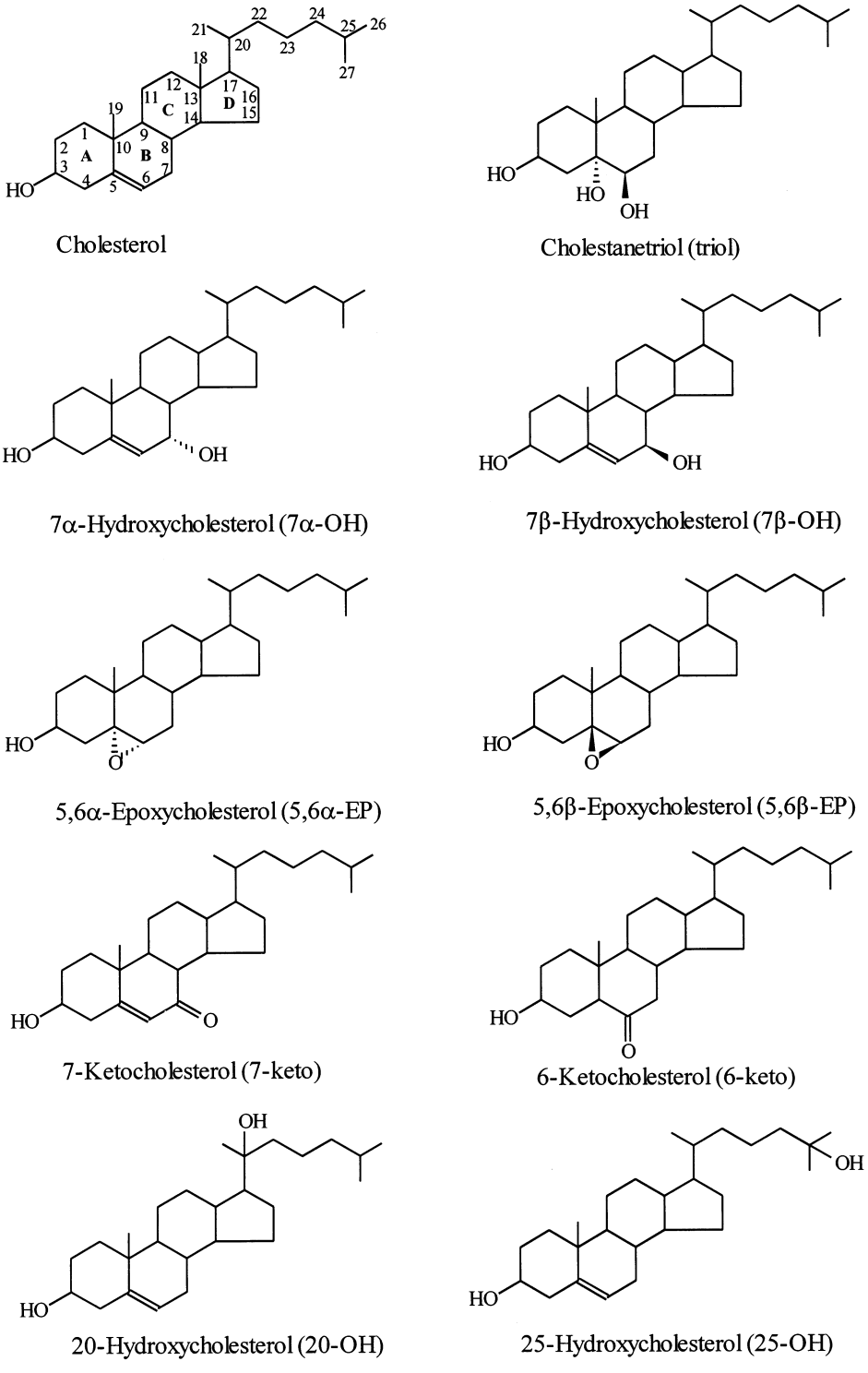

Figure 1. The chemical structures of some COPs.

The chemical structures of these COPs and cholesterol are depicted in Figure 1.

In recent years, COPs have drawn much attention mainly because of their potential health implications. Numerous studies have shown that COPs may possess biological effects such as mutagenicity[5], angiotoxicity[6,7], carcinogenicity [8], cytotoxicity, atherogenicity and cell membrane damage[7,9-11], and inhibition of cholesterol biosynthesis[12,13]. In addition, COPs may induce atherosclerosis[7,14-16].

Foods rich in cholesterol content such as dairy products, eggs and meat products, are prone to undergo autoxidation or enzymatic oxidation and form COPs[17]. COPs can also be generated during food preparation when exposed to heat, air, light and radiation[17]. Moreover, inappropriate storage conditions will also facilitate COPs formation[2]. Generally, heat, pH, light, oxygen, water activity, and the presence of unsaturated fatty acids are the major factors that influence COPs formation during food processing or storage[4]. Due to the potential health risk of consumption of COPs-containing foods, it is important to learn more about the formation and inhibition of COPs in foods during heating or illumination. This paper deals with an overview of analysis, formation and inhibition of COPs in foods.

CHOLESTEROL OXIDATION

The susceptibility of cholesterol to oxidation has been recognized and investigated for more than a century[18]. However, the formation pathways of certain COPs still have not been fully clarified. Cholesterol oxidation is reported to be similar to that of lipid oxidation[19], i.e., it can be initiated in the presence of oxygen (air) at elevated temperatures or under light resulting in autoxidation or photooxidation. The autoxidation of unsaturated fatty acid such as oleic acid can be initiated at C-8 or C-11, while the cholesterol autoxidation can be initiated at C-7. Because of the ring structure of cholesterol, the oxidation products of lipids can be more complex than cholesterol. Since cholesterol-containing phospholipids, fatty acids and cholesterol are associated closely as the integral part of the lipid bilayer of the cell membrane, the hydroperoxides derived from oxidation of unsaturated fatty acids are believed to play an important role in facilitating cholesterol oxidation[19]. Cholesterol consists of four fused rings, an aliphatic side chain branched to the D ring at C-17, a hydroxyl group attached to the A-ring at C-3, and a double bond between C-5 and C-6 of B ring (Figure 1). This double bond makes both C-4 of A-ring and C-7 of B ring on the same plane. One may expect that both the C-4 and C-7 position should have an equal opportunity for an oxidative attack to occur. However, C-7 is indeed a common position for oxidants to react. In contrast, the attack rarely occurs at C-4 because of the possible shielding effect provided by the neighboring hydroxyl group at C-3 and the trialkyl substituted C-5[18]. Both the 20- and 25-C of the aliphatic side chain are at a tertiary position, and are, therefore, more susceptible to oxidative attack than the other carbons[19].

I. *Autoxidation*

Cholesterol oxidation can be initiated by abstraction of hydrogen, predominantly at C-7, followed by the addition of an oxygen molecule, which leads to formation of 7α-hydroperoxycholesterol (7α-OOH) or 7β-hydroperoxycholesterol (7β-OOH), the primary oxidation product during heating (Figure 2). The reduction of 7α- and 7β-OOH further results in formation of 7α- and 7β-OH, which are widespread in foods[20]. Both isomeric 7-OOH can also undergo dehydration during heating to form 7-keto[21,22], which is also a major product of cholesterol autoxidation in the food system. In addition, 7-keto can be formed through dehydrogenation of the isomeric 7-OH in the presence of radicals[23]. Under basic conditions, 7-keto can be converted to 3,5-cholestadien-7-one and other compounds[18]. For 5,6α-EP or 5,6β-EP, it can be formed when cholesterol is subjected to autoxidation at pH 8 for 3 hrs[24]. However, with the direct attack of cholesterol by singlet or triplet oxygen, only hydroperoxides, but not epoxides, were formed[18]. During autoxida-

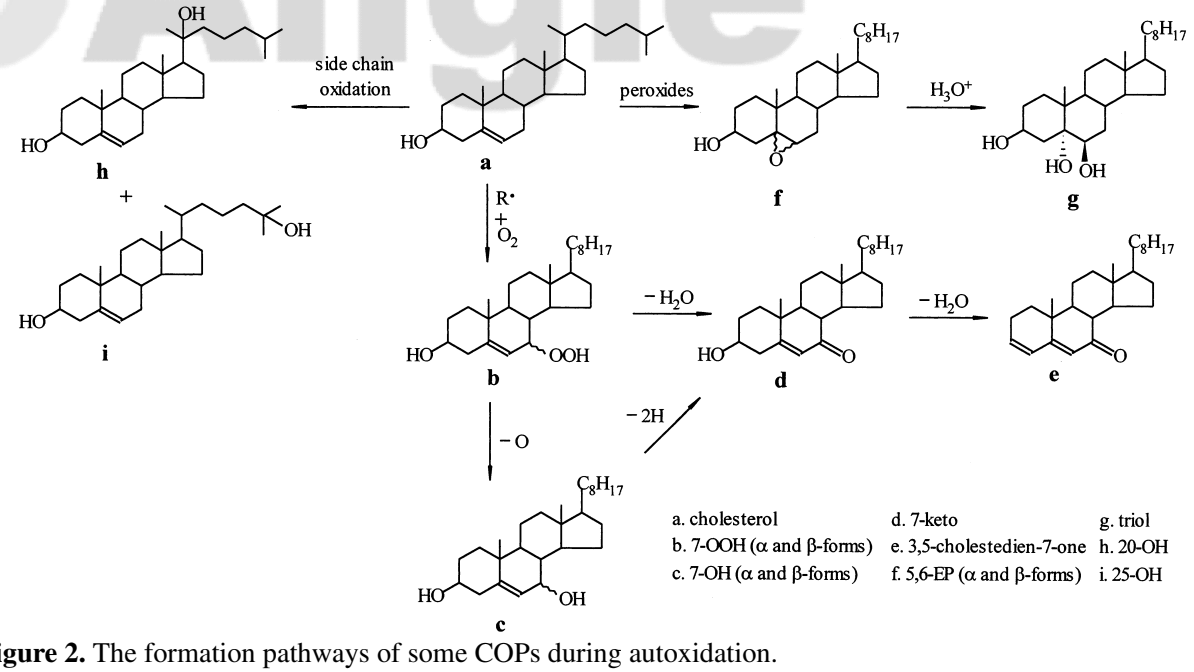

Figure 2. The formation pathways of some COPs during autoxidation.

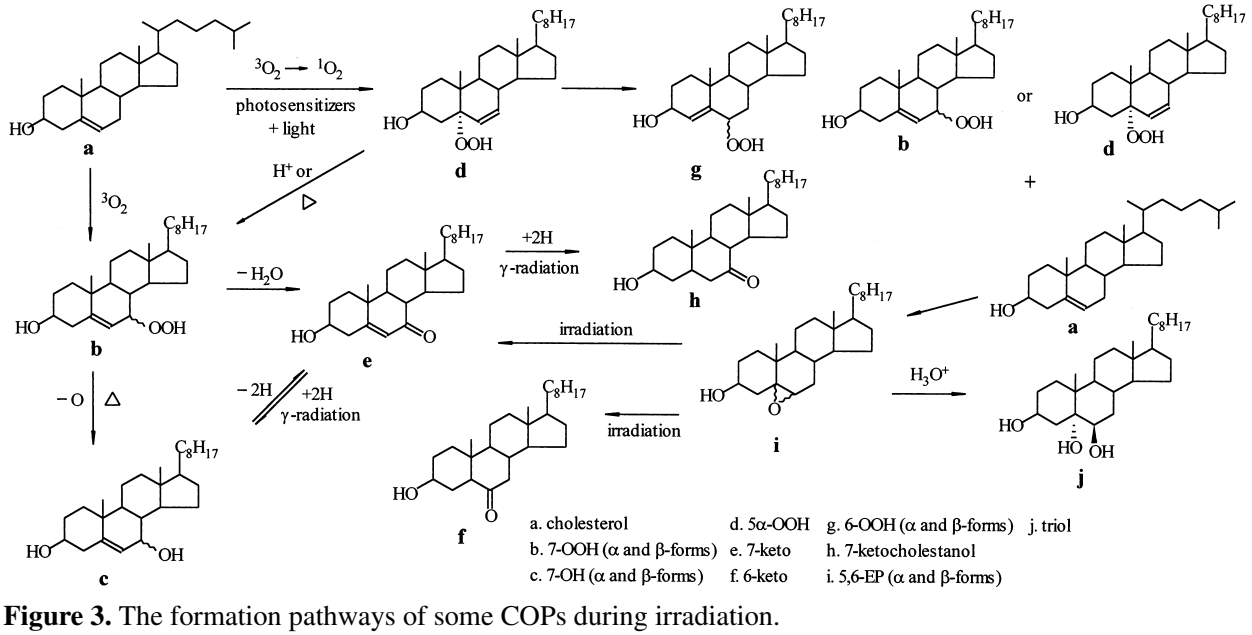

Figure 3. The formation pathways of some COPs during irradiation.

tion, the epoxides are formed when cholesterol is in a crystalline state, in solution or in dispersion [18]. In addition, the interaction between cholesterol and 7-OOH or 5-hydroperoxy-6-ene in chloroform causes formation of 5,6-EP in a minor amount. Maerker and Bunick[25] reported that the ratio of 5,6α-EP/5,6β-EP was influenced by pH of the dispersion, since β-epoxide was hydrolyzed faster than α-epoxide. When cholesterol was dispersed in sodium stearate at pH 8, 5,6-EP was observed[24]. The isomeric 5,6-EP can be hydrated under an acidic condition to form triol, which is reported to be the most toxic COPs [18].

The side chain oxidation occurs in the solid phase or in the crystalline form of cholesterol. The oxidative attack at tertiary C-20 and C-25 position generates 20-OOH and 25-OOH, respectively. These hydroperoxides can be further degraded to

20-OH and 25-OH, which are quite stable and can sustain consecutive heating at 100°C for 6 months[26]. However, this type of oxidation is not observed in solution or in aqueous dispersion[18]. The formation pathways of some COPs are summarized in Figure 2.

II. *Photooxidation*

Photosensitizers such as chlorophyll and hematoporphyrin can absorb energy in the form of radiation and transfer it to the triplet oxygen so that the more active singlet oxygen is formed. The singlet oxygen then reacts with the double bond of the B ring of cholesterol, resulting in migration of one double bond and formation of 5-OOH[27]. The 5-OOH can be further converted to the more stable 7-OOH or 6-OOH, which are present in minor amount. Irradiation of 7-keto in aqueous dispersion results in formation of 7-ketocholestanol, indicating that hydrogenation may occur through the interaction between 7-keto and the radiolysis products of water[28]. However, with increasing light intensity, both isomeric 5,6-EP and 7-OH can be further converted to 6-ketocholesterol (6-keto) and 7-keto, respectively[29,30]. Interestingly, the formation of a minor amount of the isomeric 7-OH was also observed. This result implied that both 7-OH and 7-keto may be interconvertible depending on the illumination conditions[23]. The formation pathways of some COPs during irradiation are summarized in Figure 3.

COPs ANALYSIS

The analysis of COPs has been difficult because of the presence of low concentrations (ppm to ppt) in foods. Methods for COPs analysis have been developed for decades. Prior to the qualitative and quantitative steps, COPs must be separated from the apolar fractions such as triglycerides and esterified cholesterol. The so-called "clean-up" step is referred to as the extraction procedure, which separates COPs and cholesterol from other lipid-soluble compounds and enriches them for further analysis. Methods for isolation and identification of COPs have been much improved from early thin-layer chromatography (TLC) to high-performance liquid chromatography (HPLC) and gas chromatography (GC). With the support of mass spectrometry (MS) and nuclear magnetic resonance (NMR), the identification of COPs becomes more precise and accurate in current studies. The methods for COPs analyses by TLC, HPLC and GC are summarized in Table 1.

I. *Saponification and Extraction*

Saponification is a vital step, which is conducted routinely to remove triglyceride, free fatty acid and water soluble impurities during extraction of COPs from foods[31-39]. Two saponification procedures, cold and hot saponification, are often employed. Many reports have shown that high recovery can be achieved by cold saponification at 25°C [32,35-38], and the formation of COPs artifacts can be retarded. However, the saponification time is too long (18-20 h). Thus, some authors used hot saponification (56°C) to facilitate the extraction of COPs from foods[40]. Nevertheless, it has been reported that hot saponification may degrade 7-keto and isomeric epoxides to form artifacts[40,19]. To remedy these problems several authors used silica gel- or C_{18} cartridges to extract COPs from foods[41-47]. Nourooz-Zadeh and Appelqvist[48] employed a Sep-Pak silica cartridge to enrich COPs. The cartridge was washed with hexane-1,2-dichloroethane (9:1, v/v) to remove triacylglycerols, followed by 1,2-dichloroethane-methanol (1:1, v/v) to elute COPs and polar lipids. This method was rapid in removing excessive amount of lipids and the recoveries of both radio labeled ^{3}H-cholesterol and ^{3}H-triol reached 90% and 97%, respectively. Therefore, the method is suitable for lipid-rich products such as milk powders. Hwang and Maerker[30] applied a solid phase extraction (SPE) column to extract COPs from meats. The lipid extract was loaded in the column and eluted serially with hexane-ethyl acetate of 100:2 (v/v), 85:15 (v/v) and 4:6 (v/v), respectively. The first fraction contained triacylglycerol and lipid-soluble impurities, while the second fraction contained 4-cholesten-3-one, 4,6-cholestadien-3-

Table 1. Methods used for COPs analyses by TLC, HPLC and GC

Column or material for COPs separation	Mobile phase	Detection	COPs separated	I.S.[a]	Rec.[b] (%)	DL[c]	Food or STD[d]	Ref.
TLC:								
Silica gel	Diethyl ether, ethyl acetate: heptane (1:1[f]) or acetone: heptane (1:1)	25% para-toluene sulfonic acid in water	7α-OH, 7β-OH, 5,6-EP	-[e]	-	-	Butter oil, cheeses	52
Silica gel	Heptane: ethyl acetate (3:2), benzene: ethyl acetate (3:2)	50% H_2SO_4 and heated at 110°C	7α-OH, 7β-OH	-	-	-	Butter	78
Silica gel (TLC), methyl silicone column (GC)	Toluene: MeOH (5:1)	FID, MS	7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 7-keto, 20α-OH, 25-OH	Cholestane	97	-	Milk powder	48
Silica gel (TLC), silica capillary DB-5 (GC)	Hexane: ether (70:30)	FID, MS	7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 7-keto, 20α-OH, 25-OH, triol	19-Hydroxy-cholesterol, Cholestanol	64.9-118.5	0.1 ppm	Butter	37
HPLC:								
Silicic acid (μ-Porasil)	Hexane: 2-propanol (94:6)	208, 235, 256 nm, RI, NMR	7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 7-keto	-	98.5-102.9	-	Spray-dried eggs	53
Silica gel, C_{18}	Hexane: 2-propanol (24:1), ACN[g]: water (9:1)	212, 230, 284 nm	7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 7-keto, 25-OH, cholesta-3,5-dien-7-one, cholest-4-en-3-one, cholest-4-en-3,6-dione, cholesterol	-	-	40 ppb	STD	51
Silicic acid	Hexane: 2-propanol (100:3)	210 nm, RI	7-keto, 20α-OH, 25-OH, 5,6α-EP, 5,6β-EP, 7α-OH, 7β-OH, 3,5-cholstadiene, 3,5-cholestadien-7-one, cholesterol	-	-	-	STD	54
Nucleosil NO_2	Hexane- propanol or hexane-butanol gradient	206 nm	7-keto, 20α-OH, 25-OH, 7α-OH, 7β-OH, 4β-OH	-	-	-	STD	58
Silica gel (LiChrosorb Si 60)	2-Propanol: hexane (2:98)	RI, MS	5,6α-EP, 5,6β-EP	-	-	5 μg	Whole egg, egg yolk powder	41
C_{18} (μ-Bondapak)	ACN: water (9:1)	RI, 212 nm	7α-OH, 7β-OH, 5,6-EP, triol, cholesterol	-	72-96	3 ppm	Cheese, butter oil	52
CN (Chrospher), C_{18} (Phenomenex)	Hexane: 2-propanol (95:5), ACN-MeOH gradient	212, 238, 280 nm, RI	7β-OH, 5,6α-EP, 5,6β-EP, 6-keto, 7-keto, 25-OH, triol, cholesta-4,6-dien-3-one	6-Keto-cholesterol	50.2-111.6	10 ppb, 10 ppm	STD	39
Silicic acid (μPorasil)	2-Propanol: hexane (7:93)	208 and 233 nm	7-keto, 7α-OH, 7β-OH	7-Keto-pregnenolone	98-104	-	Beef, liver sausage, fast foods, pancake mix, health foods	44

Table 1. Continued

Column or material for COPs separation	Mobile phase	Detection	COPs separated	I.S.[a]	Rec.[b] (%)	DL[c]	Food or STD[d]	Ref.
Silica gel (LiChrosorb Si 60)	Hexane: 2-propanol (100:10)	RI	7α-OH, 7β-OH	-	-	-	Egg nog mix	59
CN (LiChrospher 100 CN)	Hexane: 2-propanol (95:5)	RI	7-keto, 7α-OH, 7β-OH	-	-	-	STD	23
Silicic acid (Altex)	2-Propanol: hexane (2.5: 97.5)	205 nm	25-OH	-	58.4-100.8	-	STD, egg yolk	42
GC:								
Phenyl silicone (DB-5)		FID	5,6α-EP, 5,6β-EP, 7-keto, 4-cholesten-3-one, 4,6-cholestadien-3-one, 4-cholestene-3,6-dione	Desmosterol	78.3-88.7	10 ppb	Meats	30
Silica capillary (DB-1)		MS	7α-OH, 7β-OH, 7-keto, 25-OH, triol	5α-Cholestane	33.8-97.9	ppb	Milk powder	4
Phenyl methyl polysiloxisane		FID, MS	7α-OH, 7β-OH, 19-OH, 20α-OH, 25-OH, 5,6α-EP, 5,6β-EP, 7-keto, 3β,5-dihydroxy-5α-cholestan-6-one, triol	Sitosterol oxide	85.8-107	-	Salame, cheese, pork link, liverwurst	49
Phenyl-silicone (HP Ultra 2)		FID	5,6α-EP, 5,6β-EP, 7-keto	6-Keto-cholestanol	62.0-96.8	-	Raw meat, chicken	47
Silica capillary (DB-1)		MS	7α-OH, 7β-OH, 5,6α-EP, 7-keto	5α-Cholestane	72.3-101.2	-	Tallow	32
Silica capillary (DB-1)		FID, MS	7α-OH, 7β-OH, 5,6α-EP, 7-keto, triol	5α-Cholestane	-	-	Freeze-dried pork	34
Silica capillary (DB-1)		FID, MS	7α-OH, 7β-OH, 25-OH, 5,6α-EP, 5,6β-EP, 7-keto, triol	5α-Cholestane	-	-	Cheese	36
Silica capillary (DB-5)		FID, MS	7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 7-keto, 20-OH, 25-OH, triol	19-Hydroxy-cholesterol	-	-	Meat products	62
Methyl silicone		FID, MS	7β-OH, 7-keto, 5,6α-EP, 5,6β-EP, 25-OH, triol	5α-Cholestane	-	0.1 ppm	Fish products	63
Phenyl silicone (HP Ultra 2)		FID	7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 7-keto	6-Keto-cholestanol	-	-	Food oils	65
Silica capillary (DB-1)		FID, MS	7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 25-OH, 7-keto, triol	6-Keto-cholestanol	-	500 pg	Dried powdered egg mix	43
Cross-linked methyl silicone (Ultra Hp)		FID, MS	7α-OH, 7β-OH, 7-keto, 5,6α-EP, 5,6β-EP, 25-OH, 20α-OH	Cholestane	-	0.1 ppm	Lard, bacon	74

Table 1. Continued

Column or material for COPs separation	Mobile phase	Detection	COPs separated	I.S.[a]	Rec.[b] (%)	DL[c]	Food or STD[d]	Ref.
Methyl silicone (ULBONHR-1)		FID, MS	7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 7-keto, triol	5α-Cholestane	-	-	Fish products	72
Dimethyl polysiloxane (Rtx-1)		FID	7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 25-OH, 20α-OH, 7-keto, triol	-	54.1-102.3	1 ppm	Dried squid	73
Cross-linked methyl silicone (Ultra Hp)		FID, MS	7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 25-OH, 20α-OH, 7-keto, triol	Cholestane	95	0.2 ppm	Dehydrated egg yolk	70

[a] I.S.: Internal standard.
[b] Rec.: Recovery.
[c] DL: Detection limit.
[d] STD: COPs standard.
[e] -: Data not available.
[f] Values in parentheses represent volume by ratio.
[g] ACN: Acetonitrite.

one and 4-cholestene-3,6-dione. The other COPs, including α-epoxide, β-epoxide and 7-keto, were eluted in the third fraction. Penazzi *et al.*[1] used an SPE florisil cartridge to purify 7-keto in several foods. The sample-loaded cartridge was washed with 2-propanol-heptane (2:98, v/v) to remove triacylglycerols and cholesterol, and 7-keto was eventually recovered by acetone. Also, the authors used an SPE silica cartridge to isolate 7-keto from the same food items. The cartridge was first washed by hexane-diethyl ether (8:2, v/v) to remove impurities, followed by elution of 7-keto by two solvents, hexane-diethyl ether (1:1, v/v) and methanol (100%). The last two fractions containing enriched 7-keto were pooled for further analysis. The application of an SPE cartridge has been considered an easier and faster method than the cold saponification method[1]. To purify COPs from milk powders, Dionisi *et al.*[4] separated COPs from the unsaponificable components using an aminopropyl-SPE cartridge, with removal of impurities by hexane/ethyl acetate (95/5, v/v) and hexane/ethyl acetate (90/10, v/v). Finally, COPs were recovered by acetone for further analysis. The extraction efficiency of COPs by a Sep-Pak C_{18} cartridge or a Sep-Pak silica gel cartridge has been evaluated by Chen and Chen[39], who reported that the former is more effective than the latter. It has been well established that the application of a cartridge for extraction of COPs is more efficient and able to prevent the formation of COPs artifacts. However, some impurities such as cholesterol, cholesterol ester, free fatty acid and triacylglycerol may be coeluted and thus interfere with the subsequent separation and identification. To solve this problem, the selection of an appropriate solvent system to remove impurities without affecting the elution of COPs is extremely important.

Nourooz-Zadeh[45] applied an enzymatic procedure to release the esterified cholesterol oxides. Although this practice can minimize degradation of the labile COPs to form artifacts, it is a time-consuming process since several TLC and solid-phase extraction steps are necessary to enrich COPs; therefore, this method is not practical for

routine analysis. Schmarr *et al.*[49] developed a transesterification method to analyze polar COPs such as hydroxycholesterols and triol. The transesterification method was conducted under a mild condition, which limited formation of artifacts. Also, this method prevents formation of emulsions. Following transesterification, the samples were subjected to an SPE column and a stepwise elution with several solvents to separate COPs from the other less polar compounds. Results showed that high recovery (> 85 %), no artifact formation and short analysis time were achieved. This method apparently shows several advantages over the other methods that have been discussed previously, however, more studies with a variety of food items are required to make a comparison so that a final conclusion can be made.

The application of different extraction techniques may end up with variations in data, which may have an impact on the interpretation of results. To remedy this problem, Dionisi *et al.*[4] compared two major extraction procedures; one involved a preliminary fat extraction followed by a saponification step, while the other involved a direct saponification step. The unsaponificable components and COPs were then isolated for further analysis from milk powder samples. The latter method was found to be superior to the former method in minimizing artifact formation, analysis time and consumption of solvent.

For many researchers, the development of a method with simplicity, quickness and accuracy for routine analysis of COPs is an ultimate goal. Penazzi *et al.*[1] developed two methods for analysis of 7-keto in egg noodles, biscuits, sweet snacks, grated cheeses, whole-milk powders and whole-egg powders. The first method was carried out on a SPE florisil cartridge for enrichment of 7-keto, followed by a normal-phase HPLC analysis, while the second method used an SPE silica cartridge for enrichment of 7-keto and subsequent analysis by a reversed-phase HPLC column. The recoveries were higher than 99% for both methods. In addition, good sensitivity and reproducibility were achieved. No degradation of 7-keto was observed, mainly because of the exclusion of the saponification step during extraction. Despite these advantages, both methods are only applicable for free 7-keto analysis, but not for its esterified form. Moreover, these methods only focused on single COP analysis, which may limit their application to the other COPs.

Artifacts are a major concern for the precise quantification of COPs. Even though many methods have been used to quantify COPs, the results have shown discrepancies. For the purpose of monitoring formation of COPs artifacts, Rose-Sallin *et al.* [50] added the deuterium-labeled cholesterol ([$^2H_7$]Cholesterol) standard into the samples to determine the artifacts formed during the clean-up steps. The labeled COPs, which were derived from the labeled cholesterol and were identified by GC-MS, represent the artifacts. The artifacts were then subtracted from the unlabeled COPs and allowed to measure artifact-free COPs. This procedure may prevent interference of artifacts, however, it can be an expensive approach because of the high cost of the labeled cholesterol standard.

II. *TLC Analysis*

Thin-layer chromatography (TLC) has been used previously for separation of COPs[22,25,51,59,78]. Although TLC can readily separate some side-chain and B-ring hydroxycholesterols, the separation of cholesterol hydroperoxides has been difficult[22]. Nevertheless, TLC can be used to confirm the identity of COPs based on their distinctive color development after spraying with sulfuric acid and observation under UV light[19,25]. Finocchiaro *et al.*[52] analyzed COPs by using an activated silica gel TLC plate, which was developed successively with diethyl ether and ethyl acetate-heptane (1:1) to separate 7α-OH, 7β-OH and 5,6-EP. The plate was then dried and sprayed with 25% aqueous para-toluene sulfonic acid for color development. The major drawback of this method is that it is not able to separate the isomers of 5,6-EP. Also, the method is tedious and not appropriate for routine analysis. Moreover, the quantitation of COPs based on the spot areas on the TLC plate is not as accurate as that by HPLC

or GC. Because of the limitation of this technique, the application of TLC alone for COPs analysis may end up with less reliable results. Nevertheless, TLC is a useful tool for COPs purification. Nourooz-Zadeh and Appelqvist[48] applied a preparative TLC technique to separate COPs from milk powders. A mobile phase of toluene-methanol (5:1, v/v) was used to elute cholesterol and COPs, which were then scraped off and analyzed by GC. The COPs including 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP and 7-keto on the TLC plate were sprayed with 0.05% 1,2-dichlorofluorecein and viewed under UV light (365 nm). Compared to the work of Finocchiaro *et al*.[52], this method permits the use of a simple irrigation system and is able to resolve both 5,6α-EP and 5,6β-EP. Pie *et al*.[37] also used the same technique to separate COPs but with a different solvent system (hexane-ether, 70:30, v/v). The COPs on the TLC plate were visualized by spraying with Rhodamin (Sigma, St. Louis, MO, USA) and removed for further analysis by GC-FID and GC-MS. Fontana *et al*.[53] employed a solvent system of benzene-ethyl acetate-acetic acid (60:40:1, v/v/v) to separate five COPs, 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP and 7-keto, in spray-dried eggs by TLC, and visualized by spraying the plate with 0.1% $Ce(SO_4)_2$-2N H_2SO_4. Despite these advantages, TLC is not suitable for quantitative analysis because of low sensitivity and resolution. In view of this problem, many methods such as high-performance liquid chromatography (HPLC), gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) have been developed and become the most frequently used tools for COPs analysis.

III. *HPLC Analysis*

HPLC is one of the most widely used techniques for separation of COPs and has been shown to elute COPs effectively[39,42,44,45,51,54,55,56]. Both reversed- and normal-phase columns have been applied to the separation of COPs, and the former is reported to result in higher reproducibility than the latter[51,57]. The reversed-phase column is currently used more often than the normal-phase column.

Ansari and Smith [51] developed two HPLC methods using a silica gel or a C_{18} column, respectively, to resolve 10 COPs by using an isocratic solvent system of hexane-isopropanol (24:1, v/v) or acetonitrile-water (9:1, v/v), with detection at 212 nm. However, baseline drift occurs and the separation time (60 min.) is too long. Also, some COPs are partially overlapped, and a C_{18} column provides a better resolution than a silica gel column. Tsai and Hudson[54] also developed a HPLC method to resolve 10 COPs by using a binary solvent system of hexane-2-propanol (100:3, v/v), with a flow rate at 3.0 ml/min and by refractive index detection (RI). Separation is complete within 28 min., however, several COPs are not adequately resolved. The authors also demonstrated that HPLC might resolve a variety of oxygenated derivatives of cholesterol with polar groups on the various carbon atoms of the isoprenoid side-chain. Csiky[58] further developed a gradient solvent system of hexane-propanol or hexane-butanol. Six COPs and cholesterol were resolved within 30 min., with detection at 206 nm. However, baseline drift occurs which can affect the quantitation accuracy. Tsai and Hudson[41] used HPLC to purify COPs in egg yolk by employing a solvent system of 2-propanol-hexane (2:98, v/v), with RI detection and flow rate 1 ml/min. Two COPs, 5,6α-EP and 5,6β-EP, are adequately resolved, however, the separation time (50 min.) is too long. Also, the detection limit for both is 5 μg, which is higher than that by UV detection. Finocchiaro *et al*.[52] employed a C_{18} column with acetonitrite-water (9:1, v/v) as the mobile phase. Column effluent was monitored for detection of the epoxides and triol using a RI detector, whereas the 7-OH was detected at 212 nm. Seven COPs are resolved, however, some peaks are partially overlapped. Tsai and Hudson (41) employed a normal-phase column and a solid probe MS technique to identify two purified compounds form commercial dry egg products as 5,6α-EP and 5,6β-EP. However, this method is too tedious for COPs detection. Park and Addis[44] employed a solvent system of 2-propanol-hexane (7:93, v/v) to

resolve 5 COPs within 25 min. with detection at 233 and 208 nm, and flow rate at 1 ml/min. Herian and Lee [59] used a mobile phase of hexane-isopropanol (100:10, v/v) to separate 7α-OH and 7β-OH within 8 min. with RI detection and flow rate 2.0 ml/min. Although both 7α-OH and 7β-OH are adequately resolved, the sensitivity is low and many impurities are present on the HPLC chromatogram. Kou and Holmes[42] further used a reversed-phase HPLC column to purify 25-OH, followed by quantitation on a silicic acid column, with detection at 205 nm and flow rate at 1.7 ml/min. The authors reported that the application of two columns is necessary to obtain the consistent baseline resolution of 25-OH from the other contaminating peaks. Teng[55] compared three normal-phase columns with a mobile phase of hexane-isopropyl alcohol in different proportions for separation of COPs. Results showed that the side-chain COPs were eluted in the same order for all of the columns, while the elution order of the three B-ring oxidation products changed from column to column. Eleven COPs were resolved using a μPorasil column (30 cm X 3.9 mm, 10-μm d_p), while 12 COPs were resolved using a Zorbax column (8 cm X 6.2 mm, 3-μm d_p). Chen and Chen [39] evaluated both CN- and C_{18}-columns for the separation of COPs. Hexane-2-propanol (95:5, v/v) was used as the mobile phase for the former, which resolved 8 COPs standards within 18 min. For the latter, a gradient system of acetonitrite-methanol in various proportions was used to resolve 9 COPs within 60 min. Both columns resulted in good resolution and steady baselines, but the μPorasil column may be a better choice because of the superior separation time. This study also shows that UV detection is 1,000 times more sensitive than RI detection, however, the former is not applicable for cholesterol epoxides.

IV. *GC Analysis*

Numerous GC methods have been developed to separate the various COPs in foods[1,4,32-38,41,43-45,49,50,60]. The combination of GC and the flame ionization detector (FID) provides a powerful tool to precisely quantify COPs. For cholesterol and COPs analyses, the conversion of these compounds to trimethylsilyl (TMS) ether derivatives is a frequently applied step to stabilize some of the diols[18]. Kao and Hwang[73] separated cholesterol and 8 COPs with good resolution by using a fused silica capillary (dimethyl polysiloxane) column. With the help of the purified standards, they identified and quantified several COPs in baked dried squid. The application of GC-MS with a selected ion monitoring mode (SIM) and a capillary column is generally regarded as the most rapid and sensitive mean to identify COPs[4,50,60]. By comparing the mass spectrum with the established chemical library, COPs can be easily identified with high accuracy. Park and Addis[32] used a GC-MS with a fused silica capillary column to identify and quantify 7α-OH, 25-OH, 5,6α-EP, 5,6β-EP, 7-keto and triol in heated tallow. In a later study, Park and Addis[34] examined the formation of COPs in some meat products by GC equipped with the same column. The temperature was programmed from 180°C to 250°C with a rise of 3°C/min, and cholesterol and five COPs, 7α-OH, 7β-OH, 5,6α-EP, 7-keto and triol were separated and identified within 25 min. Sanders *et al*.[36] also used a GC-MS method and a DB-1 column to separate and quantify 7α-OH, 7β-OH, 25-OH, 5,6α-EP, 5,6β-EP, 7-keto and triol in a variety of cheese powders. Pie *et al*.[62] used a 30-m fused capillary silica DB-5 column for separation of 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP, 7-keto, 20-OH, 25-OH and triol in meat products, followed by detection with GC-MS. Ohshima *et al*.[63] analyzed COPs in fish products by conversion of them to TMS-derivatives and subsequent separation and detection by GC with a flame ionization detector (GC-FID). The identification of these COPs was carried out in a quadrupole MS fitted with an electron ionization source, and six COPs, 7β-OH, 7-keto, 5,6α-EP, 5,6β-EP, 25-OH and triol were quantified. In addition, both GC-FID and GC-MS were used to identify and quantify cholesterol content in eggs[64]. Schmarr *et al*.[49] determined COPs in several food products using GC-FID and GC-MS, and a high recovery was found for the moderate polar and polar oxysterols, how-

ever, the less polar COPs may undergo partial loss by this method. The identified COPs include 7α-OH, 7β-OH, 19-OH, 20α-OH, 25-OH, 5,6α-EP, 5,6β-EP, 7-keto, 3β,5-dihydroxy-5α-cholestan-6-one and triol. Among these COPs, some (19-OH and 20α-OH) were rarely identified by HPLC. Li *et al*.[65] used both GC-FID and GC-MS to identify and quantify several COPs in the heated oils, including 7α-OH, 7β-OH, 5,6α-EP, 5,6β-EP and 7-keto. Dionisi *et al*.[4] employed a GC-MS technique to detect COPs in milk powders, and the detection limit was reported to be 5 ppb. Due to the chemical properties of COPs, most of the GC columns used are with low polarity. The columns used in various studies are listed in Table 1.

V. *Comparison of HPLC and GC-MS*

HPLC and GC-MS are the most frequently used methods for COPs analysis. HPLC is often carried out under ambient temperatures while GC is under high temperatures. Although the resolution power of HPLC is theoretically inferior to that of GC, the former can provide an ideal means for sample recovery and purification. Meanwhile, HPLC can simplify the quantification procedure, shorten the analysis time and introduce fewer artifacts[54]. HPLC is commonly equipped with an UV detector to monitor COPs, however, some double bound-free COPs such as the isomeric 5,6-EP and triol can not be detected. The narrow absorbance range of most COPs may result in interference with detection as well as limitation of the choice of solvents. In addition, the production of solvent waste by HPLC analysis poses a disposal problem. On the other hand, GC can be used to resolve geometric isomers, which can not be separated by HPLC. As mentioned, the combination of GC and MS is an effective tool for COPs analysis, which includes not only quantification but also identification. The mass spectrums derived from GC-MS assist elucidation of the chemical structures of COPs and further contribute to their identification. However, the major drawback of GC is that it may also thermally destroy cholesterol and B-ring hydroperoxide to form artifacts[38,52]. In addition, COPs need a derivatization step for GC separation, which extend the analysis time and affect the quantitation accuracy. Thus, HPLC can be more suitable to the routine COPs analysis in food samples than GC.

(To be continued in next issue)

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