Oxidation of Cholesterol by Heating

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Oxidation of pure cholesterol during heating in an air oven at high temperature was studied. Cholesterol was virtually stable during heating at 100 °C for 24 h but was unstable at temperatures above 120 °C. In the heated cholesterol preparations, a number of oxidized derivatives were detected by a combination of thin-layer chromatography and capillary gas chromatography–mass spectrometry. Major oxidized sterols were 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5α-epoxycholesterol, 5β-epoxycholesterol, cholestanetriol, and 7-ketocholesterol. Various oxidized cholesterol derivatives were produced during heating above 120 °C within a relatively short time (1 h). The composition of the oxidized products differed depending on temperature and time of heating. When cholesterol was heated at 150 °C, the production of oxidized cholesterol was maximum, and 7-ketocholesterol was the most predominant oxidized product. Heating at 120 °C also produced oxidized cholesterol to some extent, whereas only marginal amounts of oxidized cholesterols were produced at 100 °C and at 200 °C cholesterol was almost decomposed in a short time.

INTRODUCTION

Cholesterol readily undergoes autoxidation, and some 70 oxidized products are known (Smith, 1981). These oxidized cholesterols display diverse biological activities, such as disturbance of cholesterol metabolism (Kandutsch and Chen, 1978; Peng et al., 1979), cytotoxicity (Jacobson et al., 1985; Peng et al., 1985, 1991; Matthias et al., 1987; Seilan and Dubuquoy, 1990), mutagenicity (Sevanian and Peterson, 1986), and carcinogenicity (Morin et al., 1991). Recently, the existence of oxidized cholesterols in cholesterol-rich processed foods, such as egg products, has been reported (Nourooz-Zadeh and Appelqvist, 1987; Sander et al., 1989; Pie et al., 1990). Since heating is the most common process in food processing, it seems interesting to measure the oxidation products in heated foods. In fact, oxidized cholesterols were found in heated tallow (Park and Addis, 1986) and meat products (Monahan et al., 1992). Although there are a number of studies in the literature dealing with the effect of heating on cholesterol oxidation (Ryan et al., 1981; Bascoul et al., 1986; Yan and White, 1990), systematic evaluation is rather scarce.

One of the problems encountered in these studies is the method of analysis; capillary gas chromatography (GC) (Park and Addis, 1985; van de Bovenkamp et al., 1988; Pie et al., 1990) and high-performance liquid chromatography (Tsai and Hudson, 1981; Maerker et al., 1988; Csallany et al., 1989) have been adopted, but it is generally difficult to resolve individual oxidized cholesterols satisfactory. Previous studies (Bascoul et al., 1986; Ryan et al., 1981; Yan and White, 1990) on the autoxidation of cholesterol by heating with fat such as lard and tallow detected 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5α-epoxycholesterol, 5β-epoxycholesterol, cholestanetriol, and 7-ketocholesterol.

In this paper, we studied the effect of varying temperatures above 100 °C on oxidized cholesterol formation, since the heating temperature differs depending on the type of cooking, such as baking, roasting and deep-fat frying. Identification of the major oxidized cholesterols was made by capillary gas chromatography–mass spectrometry (GC–MS).

MATERIALS AND METHODS

Reagents. 5α-Cholestane, cholest-5-en-3β-ol (cholesterol), 5,6α-epoxy-5α-cholestan-3β-ol (5α-epoxycholesterol), 3β-hydroxycholest-5-en-7-one (7-ketocholesterol), cholestane-3β,5α,6β-triol (cholestanetriol), cholest-5-ene-3β,7β-diol (7β-hydroxycholesterol), cholest-5-ene-3β,20α-diol (20α-hydroxycholesterol), cholest-5-ene-3β,22(S)-diol [22(S)-hydroxycholesterol], cholest-5-ene-3β,22(R)-diol [22(R)-hydroxycholesterol], and cholest-5-ene-3β,25-diol (25-hydroxycholesterol) were purchased from Sigma Chemical Co., St. Louis, MO. Cholest-5-ene-3β,7α-diol (7α-hydroxycholesterol) and 5,6β-epoxy-5β-cholestan-3β-ol (5β-epoxycholesterol) were obtained from Steraloids, Inc., Wilton, NH. Trimethylchlorosilane (TMCS) and 1,1,1,3,3,3-hexamethyldisilazane (HMDS) were the products of Nacalai Tesque Co., Kyoto. Other reagent grade chemicals were purchased from Wako Pure Chemical, Osaka.

Heating of Cholesterol and Analysis of Oxidized Cholesterol. Cholesterol dissolved in chloroform (50 mg/mL) was placed in 10-mL tubes (10 mm × 70 mm); the solvent was evaporated under a nitrogen stream to make a thin cholesterol film and then heated for various periods of time (1, 3, 6, 12, and 24 h) and at various temperatures (100, 120, 150, and 200 °C) in an electric oven. The heated cholesterol was converted to trimethylsilyl ester by a mixture of TMCS, HMDS, and anhydrated pyridine (1:3:9 v/v/v) at room temperature, and trimethylsilyl esters were applied to capillary gas chromatography (GC) using 5α-cholestane as an internal standard.

Identification and Isolation of Oxidized Cholesterols. Heated cholesterol was applied on a sililic acid column (silica gel 60, 70–230 mesh, E. Merck, Darmstadt, 18 mm × 200 mm) and fractionated by successive elution with 50 mL of *n*-hexane, 50 mL of diethyl ether and finally 70 mL of methanol. An aliquot of the polar fraction eluted by methanol was loaded on a thin-layer plate (Kieselgel 60, 0.25-mm thickness, E. Merck) and developed in hexane/diethyl ether/ethyl acetate (50:50:50 v/v/v) (Pie et al., 1991). The plate was sprayed with 3% copper acetate–8% phosphoric acid solution (Fewster et al., 1969) and heated at 140 °C for 10 min to detect oxidized cholesterols. Another aliquot was converted to trimethylsilyl ester and applied on capillary GC and GC–MS.

Gas Chromatography Conditions. Trimethylsilyl derivatives of cholesterol and its oxidized products were separated by capillary GC (GC-7AG, Shimadzu Co., Kyoto) with flame ionization detector and C-R6A integrator. The GC conditions

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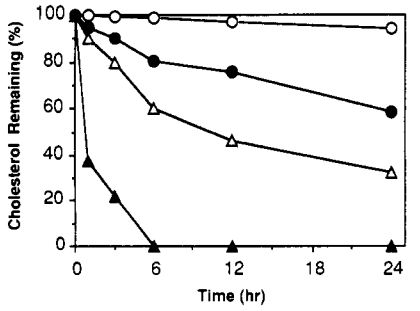

Figure 1. Stability of cholesterol during heating at various temperatures. Cholesterol was heated at 100, 120, 150, and 200 °C for 24 h in an electric oven. Symbols: ○, 100 °C; ●, 120 °C; △, 150 °C; ▲, 200 °C.

were as follows: a fused silica capillary ULBON HR-1 column (0.25 mm × 50 m, Shinwa Chemical Industries, Ltd., Kyoto) with a liquid phase thickness of 0.25 μm; oven temperature, 280 °C; injector temperature, 300 °C; flow rate of helium, 2.2 mL/min.

Mass Spectrometry. GC–MS was performed to identify the major peaks. The analysis was carried out on an Automass 50 mass spectrometer (JEOL Ltd., Tokyo) and a HP 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, PA) with a fused silica capillary DB-1 column (0.25 mm × 30 m, J&W Scientific, Folsom, CA) with a film thickness of 1.0 μm. The flow rate of helium was 1 mL/min. Samples were injected at the oven temperature of 240 °C. After injection, the temperature was raised to 300 °C (6 °C/min). The injector and interface temperature was 300 °C. Mass spectra were measured within a mass range of m/e = 30–650. Scan speed was 1 scan/s. Ionization energy was 70 eV.

RESULTS AND DISCUSSION

Stability of Cholesterol during Heating. The amounts of cholesterol remaining after heating at different temperatures are shown in Figure 1. At 100 °C, cholesterol was apparently stable. However, when heated at 120 °C and above, cholesterol was unstable and was completely degraded (carbonized) when heated at 200 °C for more than 6 h. At intermediate temperatures, 120 and 150 °C, approximately 60 and 40% of cholesterol remained unchanged, respectively. The results indicate that cholesterol is readily oxidized at high temperature, while it is rather stable at temperatures conventionally used for cooking, 100 °C for a short time. Bascoul et al. (1986) reported 25% of cholesterol was destroyed after 60 h of commercial frying. Also, oxidized cholesterol has been observed in long-term-stored foods (Nourooz-Zadeh and Appelqvist, 1987). From this point of view, attention should be paid to the autoxidation of cholesterol during storage and heating. Cooking at deep-fat-frying temperature may result in a considerable degree of oxidation and decomposition of cholesterol.

Isolation and Identification of Oxidized Cholesterols. Cholesterol was heated at 150 °C for 24 h, and the heated products were applied on a sililic acid column. The polar fraction rich in oxidized cholesterols was eluted by methanol and spotted on the TLC plate together with oxidized cholesterol standards. As shown in Figure 2, most of the oxidized cholesterol standards were separated except for 5α-epoxycholesterol and 5β-epoxycholesterol, which were not fully resolved. By comparing R_f values of oxidized cholesterol standards, each spot of the polar fraction (lane 12) was tentatively identified as follows; spot 1, cholesterol; spot 2, 20α-hydroxycholesterol; spot 3, 25-hydroxycholesterol; spot 4, 5α- and 5β-epoxycholesterol; spot 5, 7-ketocholesterol; spot 6, 7β-hydroxycholesterol; spot 7, 7α-hydroxycholesterol; and spot 8, cholestanetriol.

Figure 3 represents the capillary GC profiles of trimethylsilyl esters of oxidized cholesterol standards (A) and the polar fraction obtained after heating at 150 °C for 24 h (B). All of the major oxidized cholesterol standards were fully resolved from each other (Figure 3A). The resolutions of cholesterol and 7α-hydroxycholesterol and of 5α-epoxycholesterol and 5β-epoxycholesterol were satisfactory in contrast to previous studies (Park and Addis, 1985; Nourooz-Zadeh and Appelqvist, 1987; van de Bovenkamp et al., 1988) in which no reasonable separation was attained.

Mass spectra of oxidized cholesterol standards and each peak separated by capillary GC are shown in Figure 4. The m/e values of trimethylsilyl esters of oxidized cholesterol standards were as follows: 7α-hydroxycholesterol, 546, 456, 441, 366, and 351; cholesterol, 458, 443, 368, and 353; 7β-hydroxycholesterol, 546, 456, 441, 366, and 351; 5β-epoxycholesterol, 474, 456, 384, and 366; 5α-epoxycholesterol, 474, 456, 384, and 366; cholestanetriol, 546, 456, 403, 367, and 321; and 7-ketocholesterol, 472, 457, 382, and 367. These m/e values were identical to those reported previously (Park and Addis, 1985; Nourooz-Zadeh and Appelqvist, 1987; Pie et al., 1991). Although the capillary columns used in GC and GC–MS analyses were different, the resolution order of oxidized cholesterols was same (data not shown). On the basis of the retention time shown in Figure 3B and the m/e values of Figure 4, these peaks were identified as follows: peak A, 7α-hydroxycholesterol; peak B, cholesterol; peak C, 7β-hydroxycholesterol; peak D, 5β-epoxycholesterol; peak E, 5α-epoxycholesterol; peak F, cholestanetriol; and peak G, 7-ketocholesterol. From the GC chromatogram, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 5β-epoxycholesterol, 5α-epoxycholesterol, cholestanetriol, and 7-ketocholesterol were found to be the main oxidized derivatives produced during heating at high temperature. These oxidized cholesterols have also been observed in foods heated at lower temperatures (Fioriti and Sims, 1966).

Amount of Oxidized Cholesterol Produced during Heating. The time course of oxidized cholesterol production for 24 h of heating at various temperatures is shown in Figure 5. When cholesterol was heated at 100 °C, virtually no oxidized cholesterol was produced. The production of oxidized cholesterol was rather low when heated at 200 °C due to the decomposition. When cholesterol was heated at 120 °C, oxidized cholesterol was produced steadily up to 24 h. On the other hand, when heated at 150 °C, the amount of oxidized cholesterol reached a peak at 12 h and reduced thereafter. The peak value corresponded to approximately 0.4% of cholesterol. These observations together with the results shown in Figure 1 suggest that cholesterol and oxidized cholesterols are decomposed at high temperature. Bascoul et al. (1986) reported the formation of 15% of polar compounds in tallow heated at 160 °C. Although this observation was considerably higher than our results, it is likely that fatty acids in tallow accelerate the formation of oxidized cholesterol as compared with the case when cholesterol alone was heated as reported by Kim and Nawer (1991).

The time course of the production of major oxidized cholesterols during heating at 120 and 150 °C is shown in Figure 6. When cholesterol was heated at 150 °C, 7α- and 7β-hydroxycholesterol, 5α- and 5β-epoxycholesterol, cholestanetriol, and 7-ketocholesterol were formed. Among them, the production of 7-ketocholesterol was the highest, followed by 5α-epoxycholesterol, 5β-epoxycholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol. This result was similar to the report of Yan and White (1990). On the other hand, when cholesterol was heated at 120 °C, 7-ketocholesterol was the sole predominant oxidized cholesterol, although the level was half that observed when

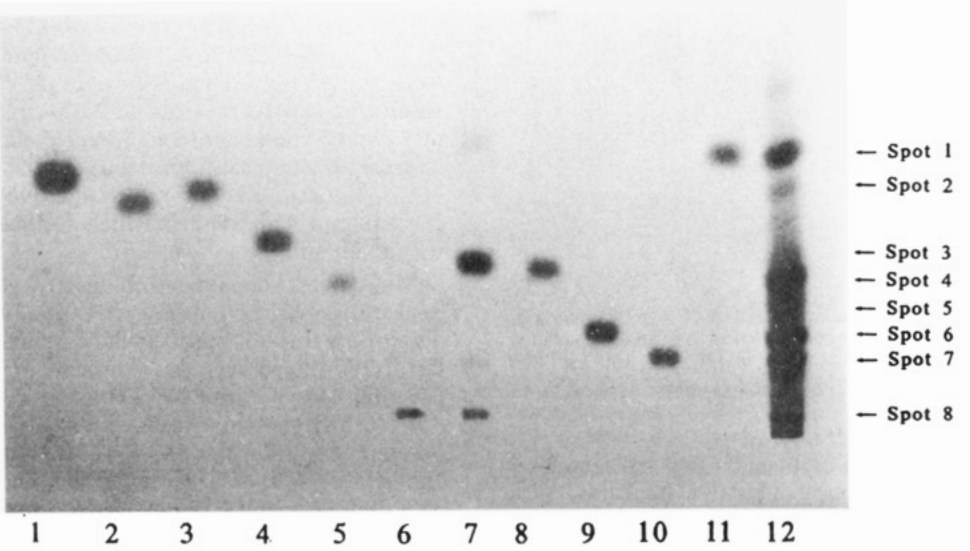

Figure 2. TLC chromatographic pattern of oxidized cholesterols. Cholesterol was heated at 150 °C for 24 h, and the polar fraction rich in oxidized cholesterol was developed in hexane/diethyl ether/ethyl acetate (50:50:50 v/v/v). Spots 1–11 were standard samples: 1, 20α-hydroxycholesterol; 2, 22(*S*)-hydroxycholesterol; 3, 22(*R*)-hydroxycholesterol; 4, 25-hydroxycholesterol; 5, 7-ketocholesterol; 6, cholestanetriol; 7, 5β-epoxycholesterol; 8, 5α-epoxycholesterol; 9, 7β-hydroxycholesterol; 10, 7α-hydroxycholesterol; 11, cholesterol; 12, oxidized cholesterol-rich fraction.

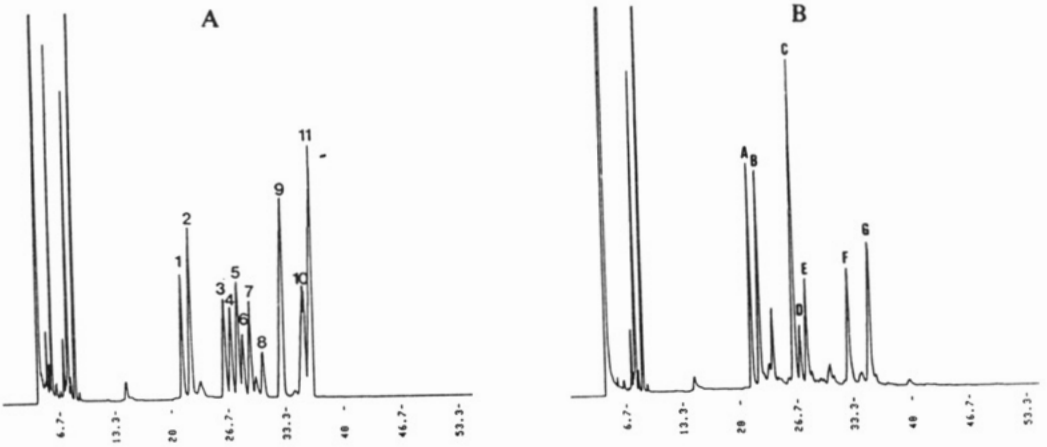

Figure 3. Gas chromatographic pattern of oxidized cholesterol standards (A) and the polar fraction rich in oxidized cholesterol (B). Cholesterol was heated at 150 °C for 24 h. The GC conditions were as described under Materials and Methods. Peaks: 1, 7α-hydroxycholesterol; 2, cholesterol; 3, 7β-hydroxycholesterol; 4, 5β-epoxycholesterol; 5, 5α-epoxycholesterol; 6, 22(*S*)-hydroxycholesterol; 7, 22(*R*)-hydroxycholesterol; 8, 20α-hydroxycholesterol; 9, cholestanetriol; 10, 7-ketocholesterol; 11, 25-hydroxycholesterol.

heated at 150 °C, and the formation of other oxidized cholesterols was marginal. Thus, the autoxidation of cholesterol may occur in a short time when heated at temperatures above 120 °C. Nourooz-Zadeh and Appelqvist (1988) reported that 7-ketocholesterol was the most predominant oxidized cholesterol of milk powder stored for a prolonged time. This supports the assumption that 7-ketocholesterol is readily produced under various processes.

The present study showed the production of various oxidized cholesterols during heating at relatively high temperature in a short time. Bascoul et al. (1986) examined the autoxidation of cholesterol in tallows during deep-fat frying and found oxidized cholesterols such as cholestanetriol, 7α- and 7β-hydroxycholesterol, 7-ketocholesterol, epoxycholesterol, and 7-ketocholesta-3-5-diene. Nourooz-Zadeh and Appelqvist (1987) also found 0.2–12 ppm of oxidized cholesterols in spray-dried egg yolk, where the actual temperature may not be above 100 °C. Pie et al. (1991) reported that the production of oxidized cholesterols varied from 0.3 to 0.5% of cholesterol in minced meats, when cooked or roasted at high temperature. These values were comparable with those observed in our model experiment. Moreover, Tsai and Hudson (1985) reported that the formation of oxidized cholesterol is greater when foods were dried by direct heating with a gas burner than when dried indirectly with steam. On the contrary, no detectable oxidized cholesterols were produced during storage of egg products and mixed diets at room temperature (van de Bovenkamp et al., 1988) or of milk powder products at low temperature (Nourooz-Zadeh and Appelqvist, 1988). Therefore, care must be taken for the processed foods, particularly in products that are heated at high temperature.

More recently, Kim and Nawar (1991) and Nawar et al. (1991) reported that autoxidation of cholesterol is exaggerated when triglyceride is present simultaneously. Thus, it is likely that when fats coexist, cholesterol is oxidized even when it is not heated. In fact, our preliminary study showed the presence of oxidized cholesterols in the nonheated or moderately heated processed marine foods as in the case of other animal products. This suggests that the degree of unsaturation of food fats may influence the production of oxidized cholesterol. Therefore, further examination is needed for the influence of coexisting fats in foods.

In conclusion, the present study showed a labile propensity of cholesterol to heating. Although no significant

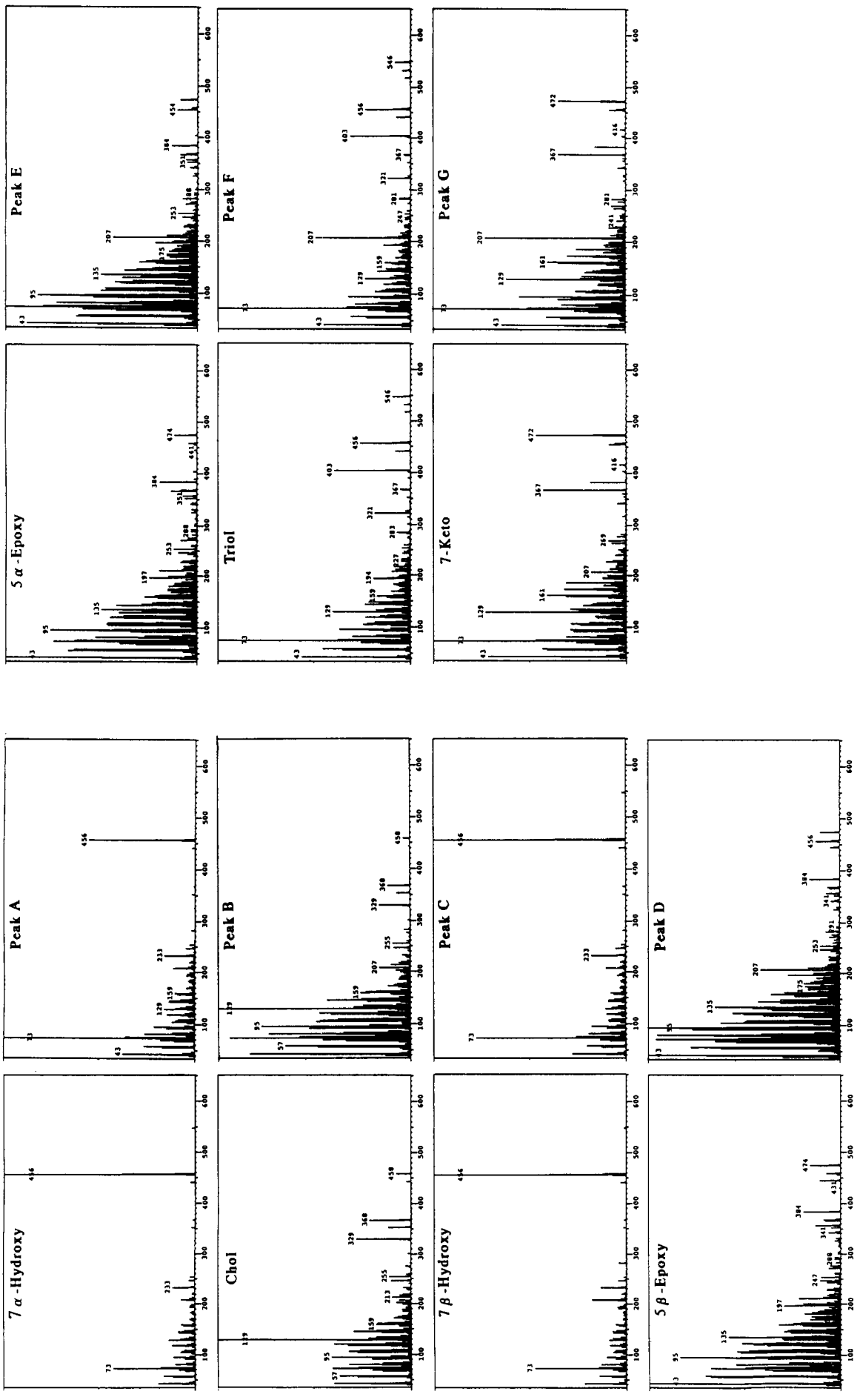

Figure 4. Mass spectra of major oxidized cholesterol trimethylsilyl ester derivatives and peaks separated by capillary gas chromatography. Cholesterol was heated at 150 °C for 24 h, and the polar fraction rich in oxidized cholesterol was separated. The GC–MS conditions were as described under Materials and Methods. For abbreviations, see text.

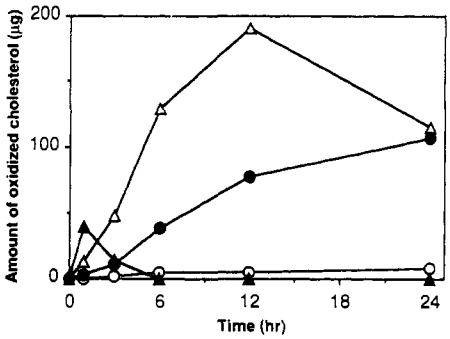

Figure 5. Time course of oxidized cholesterol production by heating at various temperatures. Amount of oxidized cholesterol was given by sum of each oxidized cholesterol quantified by capillary GC. Symbols: ○, 100 °C; ●, 120 °C; △, 150 °C; ▲, 200 °C.

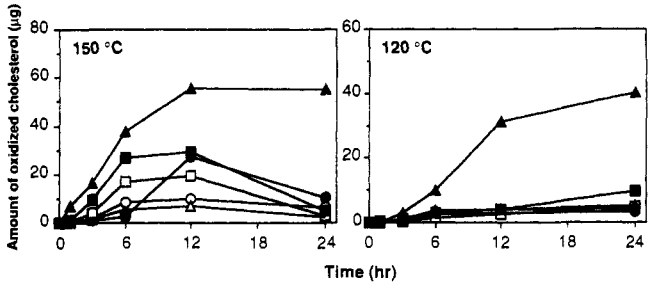

Figure 6. Production of major oxidized cholesterol by heating at various temperatures. Each oxidized cholesterol was quantified by capillary GC. Symbols: ○, 7α-hydroxycholesterol; ●, 7β-hydroxycholesterol; □, 5β-epoxycholesterol; ■, 5α-epoxycholesterol; △, cholestanetriol; ▲, 7-ketocholesterol.

amount of oxidized cholesterol itself was produced when heated at 100 °C, it seems likely that oxidation easily takes place at temperatures above 120 °C as in the case of deep-fat frying, roasting, and baking. Therefore, more attention should be paid to the oxidized cholesterols in processed and cooked foods since these products appear to have diverse untoward functions.

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