Food Chemistry **50** (1994) 53–58

Determination of cholesterol oxides in heated lard by liquid chromatography

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(Received 17 February 1993; revised version received and accepted 28 May 1993)

The presence of cholesterol oxidation products (COPS) in heated lard by liquid chromatography was studied. Lard was heated at 180°C for 0, 2, 4, 8, 10, 20, 50, 70, 100, 150 and 200 h. The various COPS present in heated lard were analysed by thin-layer chromatography and high-performance liquid chromatography. 6-Ketocholesterol was used as an internal standard to quantify all the compounds. Results implied that in most cases the COPS content increased along with the increase of heating time. The only exception was that both 5,6α-epoxycholesterol (5,6β-epoxycholestanol) and 7β-hydroxycholesterol contents increased during the first 100 h and then began to decline. Cholestan-3β-5α-6β-triol could be detected only after 20 h heating, and reached plateau as the heating time increased to 200 h The loss of cholesterol reached 70% after 150 h heating, and the amount of cholesterol lost during heating was not equal to the amount of COPS formed. Surprisingly, the highly toxic 25-hydroxycholesterol was not found.

INTRODUCTION

Cholesterol is widely distributed in foods, of which animal fats and egg products contain significant amounts. Cholesterol readily undergoes oxidation in air under a variety of conditions to yield a large number of oxidation products. To date, more than 60 cholesterol oxidation products (COPS) have been characterised (Smith, 1981), some of which are shown to be cytotoxic, mutagenic and carcinogenic (Peng *et al.*, 1979; Taylor *et al.*, 1979; Imai *et al.*, 1980; Ansari *et al.*, 1982). The major COPS in foodstuffs include the following: 25-hydroxycholesterol (25-OH), cholestan-3β-5α-6β-triol(triol), 5,6α-epoxy-cholesterol(5,6α-EP), 5,6β-epoxycholesterol (5,6β-EP), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), 7-ketocholesterol (7-keto) and cholesta-4,6-diene-3-one (4,6-diene-3-one) (Finocchiaro & Richardson, 1983). Of these COPS 25-OH and triol have been shown to be the most toxic to cultured cells and experimental animals (Kandutsch *et al.*, 1978; Peng *et al.*, 1979; Imai *et al.*, 1980; Ansari *et al.*, 1982).

Due to the presence of low concentrations of COPS in foods, the separation and quantification of COPS have been difficult. The separation of COPS has been previously achieved by thin-layer chromatography (TLC) (Ansari & Smith, 1979; Smith 1981). TLC can readily separate some side-chain and B-ring hydroxycholesterols but not cholesterol hydroperoxides (Teng *et al.*, 1973). Although the separation of COPS by TLC is lengthy and troublesome, it can still be used to confirm the identity of COPS based on distinctive colour development under UV light (Smith, 1981). In recent years many authors of published reports used gas–liquid chromatography (GLC) to separate COPS in foods (Finocchiaro & Richardson 1983; Missler *et al.*, 1985; Park & Addis, 1985, 1986*a,b*, 1987; Sander *et al.*, 1989; Nourooz-Zadeh, 1990; Yan & White, 1990). However, few reports dealt with determination of COPS in animal fats (Park & Addis, 1986*a,b*; Yan & White, 1990). Park and Addis (1986*a*) determined the formation of COPS in tallow heated at 135, 150, 165 and 180°C for 70, 144 and 216 h by capillary GLC. Only 7-keto and 5,6α-EP were found, and the amount of 7-keto formed during frying increased linearly with heating time. In a later study, Park and Addis (1986*b*) used the same technique to isolate five COPS from tallow heated at 155°C for 400 h. These COPS were identified as 7α-OH, 7β-OH, 7-keto, 5,6α-EP and 5,6β-EP. Yan and White (1990) used capillary GLC to study cholesterol oxidation in lard containing two levels of added cholesterol. 7-Keto and 5,6α-EP were the predominant COPS found, and 7α-OH, 7β-OH, and triol were formed in minor amounts. In addition, the authors suggested that thermal degradation of cholesterol may occur during heating. Although GLC was able to resolve some B-ring oxidation products and geometrical isomers, it may also thermally destroy cholesterol and B-ring hydroperoxide to form artifacts (Finocchiaro *et al.*, 1984; Yan & White, 1990).

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Food Chemistry 0308-8146/94/$07.00

High-performance liquid chromatography (HPLC) is a more gentle method to separate COPS than GLC. Although the separation power of HPLC is theoretically inferior to that of GLC, HPLC can still provide an ideal means for sample recovery and purification. Interestingly, in another study Tsai and Hudson (1981) reported that HPLC not only provided superior resolution of COPS over TLC and GLC, but also simplified the quantitation procedure and introduced fewer artifacts. The objective of this study was to determine the loss of cholesterol and the formation of COPS in lard during heating by liquid chromatography.

MATERIALS AND METHODS

Heating of lard

Commercial deodorised lard was purchased from a local supermarket. Approximately 1·5 kg lard was melted at 65°C in a water bath and then poured into a fryer. Lard was continuously heated at 180°C for 0, 2, 4, 8, 10, 20, 50, 70, 100, 150 and 200 h. After heating, about 100 g lard was intermittently removed from the fryer and cooled in ice water immediately for 3 min. Then the lard was stored at −20°C until analysed.

Materials

Cholesterol and 10 COPS standards, 25-OH, triol, 5,6α-epoxycholesterol (5,6α-EP), 5,6β-epoxycholesterol (5,6,β-EP), 7α-OH, 7β-OH, 6-ketocholestanol (6-keto), 7-keto, cholesta-3,5-diene (3,5-diene) and 4,6-diene-3-one, were purchased from Sigma Chemical Co. (St. Louis, MO, USA). 6-Keto was used as an internal standard. HPLC grade solvents such as hexane, 2-propanol, methanol, water, and ethyl acetate were purchased from Merck (Taiwan) Ltd and filtered through a 0·2 μm membrane filter under vacuum prior to use. The silica gel 60 TLC plates (20 × 20 cm) with a thickness of 300 μm were made by a CAMAG spreader (Muttenz, Germany). The TLC plates were activated at 110°C for 2 h prior to use. The spraying reagent, *N,N*-dimethyl-*p*-phenylenediamine, was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA).

Instrumentation

The HPLC instrument consisted of a Jasco 880-PU pump with Jasco 830 RI detector and SIC chromatocodor 12 integrator (Spectroscopic Co., Japan). An Advantec SF-2120 fraction collector (Toyo Roshi Co., Japan) was used to collect eluates from the column. A Lichrospher stainless-steel cyano-bonded column (25 cm × 4·6 mm i.d.) packed with 5 μm particles (Merck Co., Taiwan) was used. Sep-Pak C_{18} cartridges were obtained from Water Associates (Milford, MA, USA). A sensitivity of 0·32 AUFS and a solvent system of hexane/isopropanol (95/5 (v/v)) pumped at a flow rate of 1·0 ml/ min were used.

Extraction of COPS from lard

The application of the Sep-Pak C_{18} cartridge to extract COPS from lipid extract has been found to be more time-saving and to use less solvents for removal of triacylglycerol and cholesterol (Kou & Holmes, 1985). Kou and Holmes (1985) also found that the Sep-Pak C_{18} cartridge was superior to a silica Sep-Pak cartridge in purifying COPS from the lipid extract (Kou & Holmes, 1985). In view of this, the application of the Sep-Pak C_{18} cartridge to extract COPS from lard was used in this study. A 1 g sample of lard containing 100 μg internal standard 6-keto was dissolved in 1 ml ethyl acetate/methanol (1/1 (v/v)) to reduce the viscosity of lard and then applied on a Sep-Pak C_{18} cartridge (3 ml of volume with 500 mg of packing material). The cartridge was prewashed with 10 ml distilled water followed by 10 ml methanol before sample application. Most triacylglycerol and cholesterol were eluted with 1 ml ethyl acetate/methanol (1/1 (v/v)). COPS and the residual impurities were eluted with 10 ml methanol and 6 ml ethyl acetate/methanol (1/1 (v/v)), and the solvent was removed by a rotary evaporator under vacuum at 40°C. The residue was dissolved in 100% ethyl acetate for TLC and hexane/isopropanol (95/5 (v/v)) for HPLC.

Separation of COPS by TLC

A solvent system of hexane/ethyl acetate/methanol (70/35/10 (v/v/v)) was developed to separate cholesterol and seven COPS, which included 3,5-diene, 4,6-diene-3-one, 5,6α-EP, 7-keto, 25-OH, 7β-OH and triol. Development of TLC plates was carried out in a glass tank lined with filter paper and equilibrated for 30 min with 120 ml of hexane/ethyl acetate/methanol (70/35/10 (v/v/v)) prior to development. A mixture of cholesterol and seven COPS standards, a portion of extract from lard, and an eluate collected from the HPLC column were applied to the TLC plate with a micropipette. The chromatograms were developed over a distance of 16 cm for about 30 min at ambient temperature. Separated spots were visualised under UV light at 254 nm for detection of COPS containing two or three double bonds such as 3,5-diene, 4,6-diene-3-one and 7-keto, followed by spraying with 10% *N,N*-dimethyl-*p*-phenylenediamine and drying at 110°C for 5 min in an oven for colour development. Then the chromatoplates were further visualised under UV light at 366 nm. Identification of cholesterol and COPS in lard was confirmed by comparison of colour and R_F value with standards.

Separation of COPS by HPLC

Internal standard 6-keto (100 μg) was added to lard for extraction. The crude extract dissolved in hexane/isopropanol (95/5 (v/v)) was filtered through a 0·2 μm

membrane filter. Portions of the extract (10 μl) were injected to the HPLC chromatograph. A binary solvent system of hexane/isopropanol (95/5 (v/v)) pumped at a flow rate of 1·0 ml/min was used. The eluate was monitored with a RI detector with a sensitivity at 0·32 AUFS. The cholesterol and various COPS were identified by comparing retention times of separated peaks with reference standards and co-chromatography with added standards. Suitable fractions of COPS were collected and subjected to TLC analysis for further identification. The concentrations of cholesterol and COPS were calculated using the ratio of peak area of cholesterol and COPS over the peak area of the internal standard. The area ratios of cholesterol and COPS to internal standard were determined at four different concentration ratios of 0·5, 1·0, 1·5 and 2·0. Then, the area ratios were plotted against concentration ratios to obtain a graph. The mean of concentrations of cholesterol and COPS was calculated from the data of duplicate analyses.

RESULTS AND DISCUSSION

Table 1 shows the identification characteristics of cholesterol and seven COPS standards by TLC. A ternary solvent system of hexane/ethyl acetate/methanol (70/35/10 (v/v/v)) provided a clear separation of cholesterol and seven COPS standards. In general, the chromatographic behaviour (R_F value) of cholesterol and COPS on silica gel TLC plates was based on the polarity of COPS, the amount of silanols (Si-OH) exposed on the silica gel surface and the relative solubility of solvent system to cholesterol and COPS. The following results were found, in order of increasing polarity: 3,5-diene (R_F 0·96), 4,6-diene-3-one (R_F 0·80), cholesterol (R_F 0·69), 5,6α-EP (R_F 0·51), 7-keto (R_F 0·37), 25-OH (R_F 0·30), 7β-OH (R_F 0·15) and triol (R_F 0·10). In this study 25-OH was found to be more polar than 7-keto. However, some authors used normal-phase HPLC to separate COPS and found that 7-keto was more polar than 25-OH because the elution time of the latter was shorter than that of the former (Nourooz-Zadeh, 1990; Teng, 1991). This difference in adsorption affinity of 7-keto and 25-OH should be due to the relative solubility of COPS in the solvent system used. 3,5-diene, 4,6-diene-3-one and 7-keto were visualised as black spots under UV detection at 254 nm. After spraying with 10% *N,N*-dimethyl-*p*-phenylenediamine and drying at 110°C for 5 min in an oven, cholesterol and four COPS 5,6α-EP, 25-OH, 7β-OH and triol each possessed distinctive colour. After further detection under UV at 366 nm, six COPS and cholesterol possessed characteristic colours except that no colour was observed for 7-keto.

Figure 1 shows the HPLC chromatogram of cholesterol and eight COPS standards. All compounds were adequately resolved within 17 min by employing a cyano-bonded column with an RI detection and a mobile phase of hexane/isopropanol (95/5 (v/v)) with a flow rate of 1·0 ml/min. Nourooz-Zadeh (1990) also used the same column and solvent system with a flow rate of 0·4 ml/min to resolve cholesterol and eight COPS. However, baseline drift occurred during separation and separation was accomplished in 60 min. By increasing flow rate to 1·0 ml/min it was possible to reduce baseline drift and decrease retention time to within 17 min, and thus minimised the inaccuracy in quantification of COPS. The k' values (capacity factor) for cholesterol, 4,6-diene-3-one, 5,6α-EP, 5,6β-EP, 25-OH, 7-keto, 7β-OH, 6-keto and triol were 1·51, 2·05, 2·46, 2·63, 2·76, 3·01, 3·23, 3·40 and 5·14, respectively. Since the k' value for all standards was between 1 and 10, using a cyano-bonded column to separate COPS was a good method to choose. It has been well established that solvent strength must be properly controlled ($1 < k' < 10$) so that a satisfactory separation can be achieved (Dolan, 1990). Nevertheless, 3,5-diene was not observed on the chromatogram probably because of its low polarity, which might overlap with solvent peak. 6-Keto was found to be a suitable internal standard because it does not interfere with separation of other compounds, it can be completely eluted from the column and it is chemically inert toward other compounds. Teng (1991) used a normal-phase column with

Table 1. Identification characteristics of cholesterol and COPS standards by TLC[a]

COPS	R_F[b]	254 nm[c]	After spraying with reagent[d]	366 nm[e]
3,5-Diene	0·96	Black	—	Light brown
4,6-Diene-3-one	0·80	Black	—	Green
Cholesterol	0·69	—	Light brown	Orange–red Golden yellow
5,6 α-EP	0·51	—	Light Orange—red	Blue–green
7-Keto	0·37	Black	—	—
25-OH	0·30	—	Orange—yellow	Golden yellow
7 β-OH	0·15	—	Deep blue	Yellow–green
Triol	0·10	—	Brown	Light blue–green

[a] Solvent system: hexane/ethyl acetate/methanol (70/35/10 (v/v/v)).
[b] Solvent front = 16·0 cm.
[c] Before spraying with 10% *N,N*-dimethyl-*p*-phenylenediamine.
[d] Sprayed with 10% *N,N*-dimethyl-*p*-phenylenediamine.
[e] After spraying with 10% *N,N*-dimethyl-*p*-phenylenediamine.

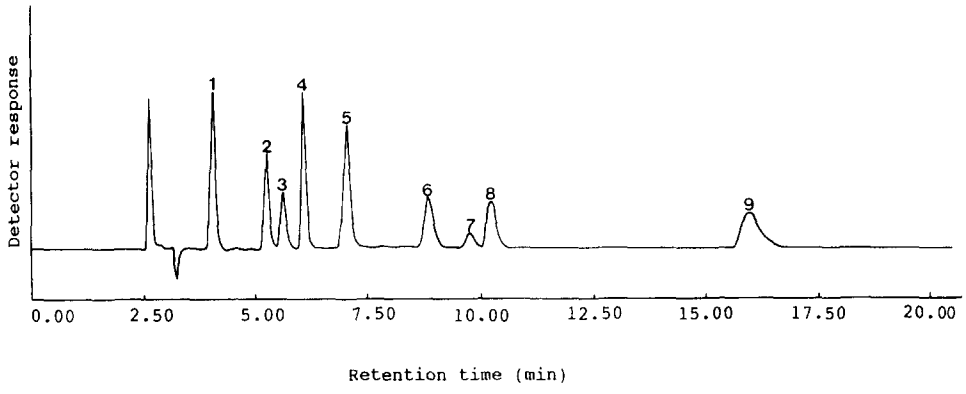

Fig. 1. HPLC chromatogram of cholesterol and eight COPS standards. Chromatographic conditions are described in the text. Peaks: 1, cholesterol; 2, 4,6-diene-3-one; 3, 5,6α-EP 4, 5,6β-EP; 5, 25-OH; 6, 7-keto; 7, 7β-OH; 8, 6-keto; 9, triol.

silica gel as the adsorbent to separate 12 COPS. Separation was accomplished in 120 min. Park and Addis (1985) used the same type of column to separate COPS in foods. Although the separation time was reduced to 25 min, only three COPs were identified. Obviously, the drastically reduced retention time of COPS when using a cyano-bonded column should be due to the fact that the interactions between COPS and Si—$(CH_2)_3CN$ was much lower than that between COPS and Si-OH. In order to demonstrate the reproducibility of this method, the COPS standard dissolved in the solvent system was continuously injected into the HPLC column five times and the coefficient of variation with respect to retention time was found to range only between 0·00 and 1·00%. Nevertheless, the retention time of COPS shifts when dealing with lard samples (see Fig. 3 below). The change in retention time may be due to environmental temperature fluctuation or column ageing.

Figure 2 shows that the cholesterol content changes during heating of lard at 180°C. After continuous heating for 200 h, the cholesterol content in lard decreased from 825 to 231 ppm. Approximately 70% cholesterol was destroyed after 150 h heating. Park and Addis (1986*a*) found that the cholesterol loss was from 40 to 45% when tallow was heated at 155°C for 300 h or 190°C for 200 h. The higher loss of cholesterol in heated lard, in comparison with heated tallow, is probably due to the fact that the former contains more residual unsaturated fatty acids (e.g. linoleic acid), which can be oxidised more easily than those present in tallow. The oxidation of linoleic acid can further accelerate the destruction of cholesterol during heating.

Figure 3 shows the HPLC chromatogram of cholesterol and COPS in lard after heating for 200 h. A total of 10 peaks was found, of which six COPS were identified. These COPS were identified as 4,6-diene-3-one, 5,6α-EP (5,6β-EP), 7-keto, 6-keto, 7β-OH and triol. Peak 3 is probably due to the presence of 5,6α-EP or 5,6β-EP as the area of this peak increased sharply when the lard sample was co-chromatographed with either 5,6α-EP or 5,6β-EP. This result seemed contradictory to that in Fig. 1, which showed that 5,6α-EP and 5,6β-EP could be adequately resolved. Apparently, this difference should be attributed to the fact that the presence of unknown COPS in heated lard might interfere with the separation of 5,6α-EP and 5,6β-EP.

Fig. 4 shows the COPS change during heating of lard. In most cases, each COPS content increased along

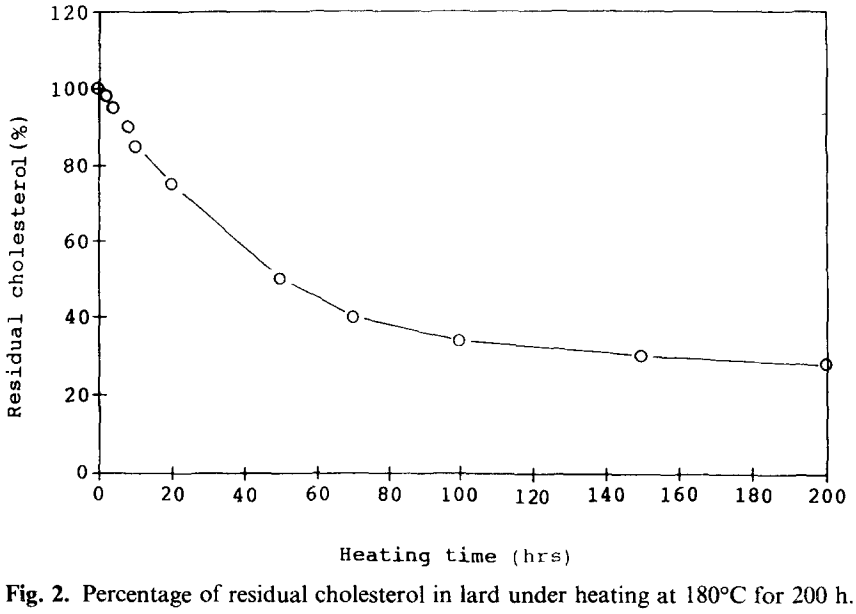

Fig. 2. Percentage of residual cholesterol in lard under heating at 180°C for 200 h.

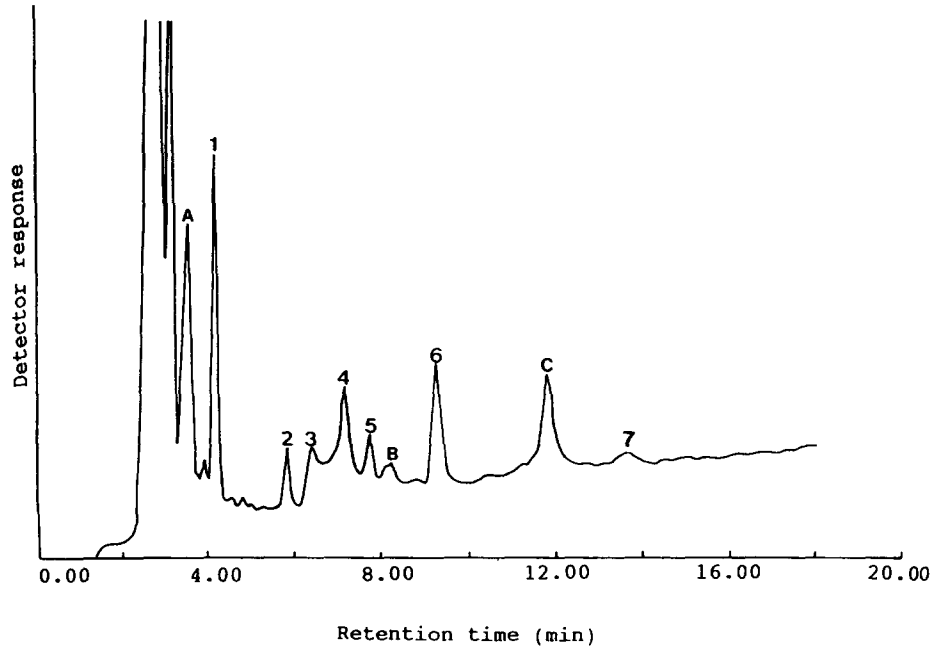

Fig. 3. HPLC chromatogram of COPS in lard under heating at 180°C for 200 h. Peaks: 1, cholesterol; 2, 4,6-diene-3-one; 3, 5,6α-EP (5,6β-EP); 4, 7-keto; 5, 7β-OH; 6, 6-keto; 7, triol; A–C unknown.

with the increase of heating time. This result implied that cholesterol was gradually converted to various forms of COPS during heating. Both 7-keto and 4,6-diene-3-one contents increased to the maximum as the heating time reached 200 h. On the other hand, both 5,6α-EP (5,6β-EP) and 7β-OH contents increased in the first 100 h and then began to decline. This result demonstrated that the epoxy-containing COPS were more susceptible to heat loss than other forms of COPS. Triol could only be detected after 20 h heating of lard, and the triol content increased thereafter and reached a maximum after 200 h heating. In addition to 4,6-diene-3-one, the COPS content change was similar to that reported by Yan and White (1990). Surprisingly, the highly toxic 25-OH was not found. The mechanism of formation of COPS from cholesterol has been well explained by Smith (1981). The author proposed that the oxidation of cholesterol was similar to that of unsaturated fatty acid. In the beginning the α-carbon by the double bond can lose a hydrogen atom to form a free radical because of heating, which then reacts with oxygen and abstracted hydrogen atom to form hydroperoxide. Hydroperoxide can be further cleaved through oxygen–oxygen linkage to form 7α-OH or 7β-OH, or it can be dehydrated to form 7-keto. In addition, heating might lower the pH of lard because of liberation of fatty acid, which in turn causes the formation of triol through 5,6α-EP or 5,6β-EP. In this experiment the amount of COPS formed (224 ppm) was not equal to the amount of cholesterol lost (594 ppm) during heating. This phenomenon may be explained as follows: (i) some COPS formed during heating of lard

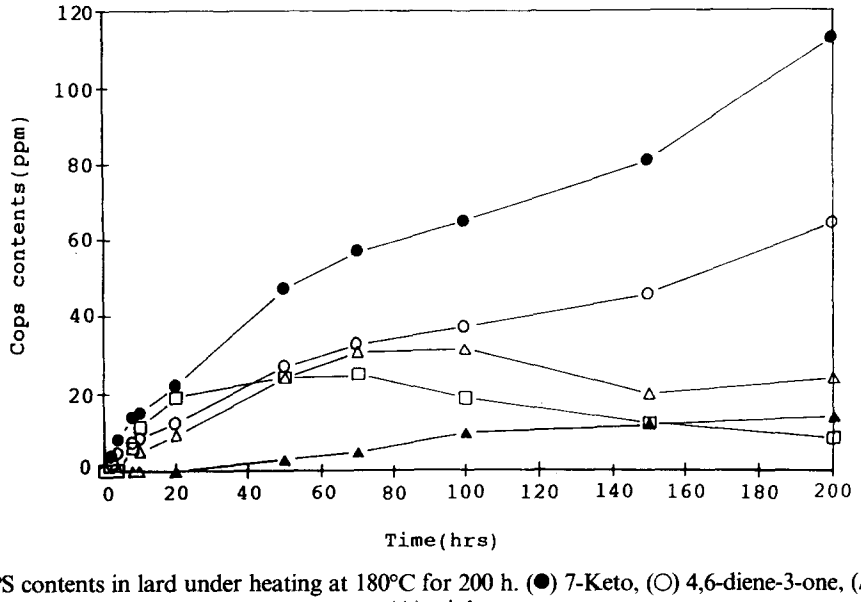

Fig. 4. Change of COPS contents in lard under heating at 180°C for 200 h. (●) 7-Keto, (○) 4,6-diene-3-one, (Δ) epoxides, (□) 7β-OH, (▲) triol.

were not identified; and (ii) some degradation products formed during heating might be volatile. In view of this, further research is necessary to identify the unknown components so that the formation mechanism of COPS from cholesterol in heated lard may be established.

ACKNOWLEDGEMENT

This study was supported by a grant (DOH81-TD-100) from the National Health Bureau, Taiwan.

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