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Evaluation of the analysis of cholesterol oxides by liquid chromatography

B.H. Chen* and Y.C. Chen

Department of Nutrition and Food Science, Fu Jen University, Taipei 242 (Taiwan)

ABSTRACT

Several extraction, separation and detection methods for cholesterol oxidation products (COPS) were developed and evaluated using liquid chromatography. The results showed that extraction of COPS from lard by cold saponification at 25°C or a Sep-Pak C_{18} cartridge resulted in higher recoveries than those given by a silica gel Sep-Pak cartridge. With HPLC, by using a cyano-bonded column and hexane–2-propanol (95:5, v/v) as the mobile phase at a flow-rate of 1.0 ml/min, and a C_{18} column with a gradient flow system with acetonitrile–methanol (55:45, v/v), it was possible to separate eight and nine COPS within 17 and 60 min, respectively. All the COPS with one, two and three double bonds could be detected by UV spectrophotometry at 212, 234 and 280 nm, respectively. UV detection had a 1000 times higher sensitivity than refractive index detection. Ten COPS were separated from heated lard using a cyano-bonded column, and five of them were identified.

INTRODUCTION

Cholesterol, an important biological compound that is widely distributed in various kinds of foods, readily undergoes oxidation in air under a variety of conditions to produce a large number of oxidation products. To date more than 60 cholesterol oxidation products (COPS) have been characterized in nature [1], some of which have been shown to be cytotoxic mutagenic and carcinogenic [2–5]. The major COPS in foodstuffs include 25-hydroxycholesterol, cholesta-3β,5α,6β-triol, 5,6α-epoxycholesterol, 5,6β-epoxycholesterol, 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol and cholesta-4,6-dien-3-one [6].

Owing to the presence of low concentrations of COPS in food, their extraction and determination have been difficult. The major problems associated with extraction of COPS are as follows: (1) most COPS are present in trace amount (ppm or ppb) in foods, which makes extraction difficult; (2) some COPS are present in esterified form, which make quantification difficult; and (3) some COPS are susceptible to oxidative loss and degradation during extraction.

Saponification has often been employed to remove neutral triacylglycerol, sterol ester and water-soluble impurities during extraction of COPS from foods [6–13]. Park and Addis [7] used cold saponification (25°C) to extract COPS from tallow and found that a 100% recovery could be achieved. Since then, this technique has been used by many workers to extract COPS from foods [10–13]. Although cold saponification could prevent the formation of COPS artifacts, the saponification time was too long (18–20 h). Some workers therefore used hot saponification to facilitate the extraction of COPS from foods [14]. However, it has been found that hot saponification might degrade 7-ketocholesterol to form artifacts [14].

In view of these problems, several workers used silica gel- or C_{18}-packed columns to extract COPS from foods [15–22]. Compared with

* Corresponding author.

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saponification methods, the application of columns for extraction of COPS was faster and could prevent the formation of COPS artifacts. However, some impurities such as triacylglycerol and free fatty acids might be co-eluted with COPS from the column and thus interfere with the separation. Also, the COPS present in esterified forms could not be determined. To solve these problems, the selection of an appropriate solvent system to remove impurities without affecting the elution of COPS was extremely important.

Methods for the separation of COPS have been developed from early thin-layer chromatography (TLC) [1,23,24] to capillary gas–liquid chromatography (GLC) [7–13,15,18,20,25,26] and high-performance liquid chromatography (HPLC) [17,19,20,24,27,28]. TLC can readily separate some side-chain and B-ring hydroxycholesterols but not cholesterol hydroperoxides [23]. Although the separation of COPS by TLC is lengthy and tedious, TLC can still be used to confirm the identity of COPS based on their distinctive colour development after spraying with acid and observation under UV radiation [1]. Capillary GLC has been found to resolve some B-ring oxidation products and geometric isomers, but it may also thermally destroy cholesterol and B-ring hydroperoxide to form artifacts [13,25]. HPLC is a milder method than GLC for separating COPS. Although the separation power of HPLC is theoretically inferior to that of GLC, it can still provide an ideal means for sample recovery and purification. Interestingly, Tsai and Hudson [27] reported that HPLC not only provided superior resolution of COPS over TLC and GLC, but also simplified the quantification procedure and introduced fewer artifacts.

The purpose of this study was to evalute several COPS extraction and separation methods using liquid chromatography. Lard was used as a reference sample for evaluation.

EXPERIMENTAL

Materials

Commercial deodorized lard was purchased from a local supermarket. Approximately 2.0 kg of lard were melted at 65°C in a water-bath and then divided into two portions of 0.5 and 1.5 kg; the former was used for method evaluation and the latter for heating.

Cholesterol and ten COPS standards, 25-hydroxycholesterol (25-OH), cholesta-3β,5α,6β-triol (triol), 5,6α-epoxycholesterol (5,6α-EP), 5,6β-epoxycholesterol (5,6β-EP), 7α-hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH), 6-ketocholestanol (6-keto), 7-ketocholesterol (7-keto), cholesta-4,6-dien-3-one (4,6-dien-3-one) and cholesta-3,5-diene (3,5-diene) were purchased from Sigma (St. Louis, MO, USA).

HPLC-grade solvents such as hexane, 2-propanol, methanol, acetonitrile, ethyl acetate, chloroform and 1,4-dioxane were purchased from Merck (Taiwan) and filtered through a 0.2-μm membrane filter under vacuum prior to use. Silica gel 60 TLC plates (20 × 20 cm) with a thickness of 300 μm were made using a Camag spreader (Muttenz, Switzerland). TLC plates were activated at 110°C for 2 h prior to use. The spray reagent N,N-dimethyl-*p*-phenylenediamine was obtained from Aldrich (Milwaukee, WI, USA).

Instrumentation

The HPLC instrument consisted of an SSI 222D digital pump (Scientific Systems, State College, PA, USA) an SSI 231 gradient controller, a Linear 206 rapid-scanning UV–Vis photodiode-array detector (Linear Instruments, Reno, NV, USA) and a Jasco Model 830 RI detector. A sensitivity of 0.32 AUFS was used. The data were stored and processed with an Axxiom 727 dual-channel chromatography system (Axxiom Chromatography, Calabasas, CA, USA). A LiChrospher CN column (25 cm × 4.6 mm I.D.) and a Phenomenex C_{18} column (25 cm × 4.6 mm I.D.) packed with material of the same particle size (5 μm) were used. Silica gel and Sep-Pak C_{18} cartridges were purchased from Waters (Milford, MA, USA).

Separation of COPS standards by TLC

A ternary solvent system of hexane–ethyl acetate–methanol (70:35:10, v/v/v) was used to confirm the identities of cholesterol and seven

COPS (3,5-diene, 4,6-dien-3-one, 5,6α-EP, 7-keto, 25-OH, 7β-OH and triol). The colour development of cholesterol and COPS under UV radiation was described in a previous paper [29].

Separation of COPS standards by HPLC

A mixture of cholesterol and ten COPS standards was dissolved in 2-propanol and hexane–2-propanol (95:5, v/v) for HPLC separation on the C_{18} column and the CN-bonded column, respectively. Concentrations of the COPS ranging from 10 to 200 ppm were prepared and injected into the HPLC system to obtain the chromatograms shown in Figs. 1, 3, 4 and 5. Acetonitrile–methanol (55:45, v/v) and UV and refractive index (RI) detection were used with the C_{18} column. The flow-rate was maintained at 0.5 ml/min for the first 25 min, then increased linearly to 3.0 ml/min within 35 min. Before the next injection the flow-rate was linearly reduced to 0.5 ml/min. The wavelength for UV detection was set at 212, 234 and 280 nm; 212 nm was used to detect most COPS, 234 for 7-keto and 3,5-diene and 280 nm for 4,6-dien-3-one. For the CN column, hexane–2-propanol (95:5, v/v) at a flow-rate of 1.0 ml/min and UV and RI detection were used. In addition, each COPS was scanned between 190 and 350 nm by employing a photodiode-array detector. The separation efficiency of these methods was determined by the capacity factor, k':

$$k' = \frac{\text{elution time of COPS} - \text{elution time of solvent}}{\text{elution time of solvent}}$$

It has been well established that k' could be affected by the solvent strength, and the best k' value should be between 1 and 10 [30]. In addition, the reproducibility of retention time was determined by injecting COPS standards five times, and the relative standard deviation (R.S.D.) of the retention time was calculated. 6-Keto was used as an internal standard.

Extraction of COPS from lard

Lard was used as a reference sample to evaluate the recovery of COPS by three extraction methods, using cold saponification, a Sep-Pak C_{18} cartridge and a silica gel Sep-Pak cartridge. HPLC was used to determine the extraction recoveries. A cyano-bonded column and a mobile phase of hexane–2-propanol (95:5, v/v) at a flow-rate of 1.0 ml/min and RI detection were used.

Cold saponification. A mixture of cholesterol, COPS standards and 100 μg of an internal standard (6-keto) was added to 1 g of melted lard in a flask. Saponification was carried out by adding 10 ml of methanolic KOH (1 *M*) to lard and allowing the mixture to stand in the dark for 18 h at room temperature. Distilled water (10 ml) was added to the solution and the mixture was poured into a separating funnel. The unsaponifiable fraction was extracted with 10 ml of hexane three times and the hexane layers were collected and combined. The combined hexane solution was further washed with 10 ml of KOH (1 *M*) and 10 ml of water, then passed through a Whatman No. 1 filter-paper containing anhydrous sodium sulphate to remove excess water. The filter-paper was washed with 20 ml of hexane to remove any residual unsaponifiable materials. The combined filtrate was then evaporated under vacuum (40°C) to remove the solvent and the residue was dissolved in an appropriate solvent for HPLC analysis.

Sep-Pak C_{18} cartridge. A Sep-Pak C_{18} cartridge was activated with 10 ml of deionized water followed by 10 ml of methanol before extraction was conducted. Melted lard (1 g) was dissolved in 1 ml of ethyl acetate–methanol (1:1, v/v) to reduce the viscosity of the lard, and a mixture of cholesterol, COPS standards and 100 μg of internal standard (6-keto) was added to the solution. The solution was poured into a Sep-Pak C_{18} cartridge, and most of the cholesterol and triacylglycerol were eluted with 1 ml of ethyl acetate–methanol (1:1, v/v). COPS and the remaining impurities were then eluted with 10 ml of methanol and 6 ml of ethyl acetate–methanol (1:1, v/v). The eluate containing COPS was then evaporated under vacuum (40°C) to remove the solvent and the residue was dissolved in an appropriate solvent for HPLC analysis.

Silica gel Sep-Pak cartridge. A silica gel Sep-Pak cartridge was activated with 10 ml of hexane before extraction was conducted. Melted lard (1 g) was dissolved in 1 ml of hexane to reduce the

viscosity of the lard, and a mixture of cholesterol, COPS standards and 100 μg of internal standard (6-keto) was added to the solution. The solution was poured into a silica gel Sep-Pak cartridge, and most of the cholesterol and triacylglycerol were eluted with 10 ml of hexane. COPS were then eluted with 20 ml of acetone, and the eluate was evaporated under vacuum (40°C) to remove the solvent and the residue was dissolved in an appropriate solvent for HPLC analysis.

Recovery data were subjected to analysis of variance (PROC ANOVA) and Duncan's multiple range test procedures of the statistical analysis system [31].

Determination of COPS in heated lard by TLC and HPLC

In order to demonstrate the best extraction and separation efficiencies of the developed methods, *ca.* 1.5 kg of lard heated at 180°C for 200 h was used as a reference sample for evaluation. COPS in heated lard were extracted using a Sep-Pak C_{18} cartridge and analysed using the cyano-bonded column with RI detection. COPS were identified by comparison of the retention times of unknowns with those of reference standards, addition of COPS standards to the sample for co-chromatography and collection of eluates for TLC analysis for further identification.

RESULTS AND DISCUSSION

LC separation and detection

Fig. 1 shows the HPLC of cholesterol and eight COPS standards using the cyano-bonded column with UV detection at 212, 234 and 280 nm. Only cholesterol and five COPS standards (4,6-dien-3-one, 25-OH, 7-keto, 7β-OH and 6-keto) were detected at 212 nm. Triol, 5,6α-EP and 5,6β-EP were not detected because of absence of π-electrons. 3,5-Diene was not separated probably because of its low polarity, so that it overlapped with the solvent peak. When the UV detection wavelength increased from 212 to 234 nm, only 4,6-dien-3-one and 7-keto were detected. In addition, 4,6-dien-3-one was the only COPS detected at 280 nm. By comparing the detector responses of each COPS on the HPLC trace, it was found that 4,6-dien-3-one has the maximum absorption at 280 nm, 7-keto at 234 nm and the other COPS containing one double bond at 212 nm.

However, the UV scanning spectra of cholesterol, 7β-OH and 25-OH were found to have maximum absorption between 200 and 205 nm

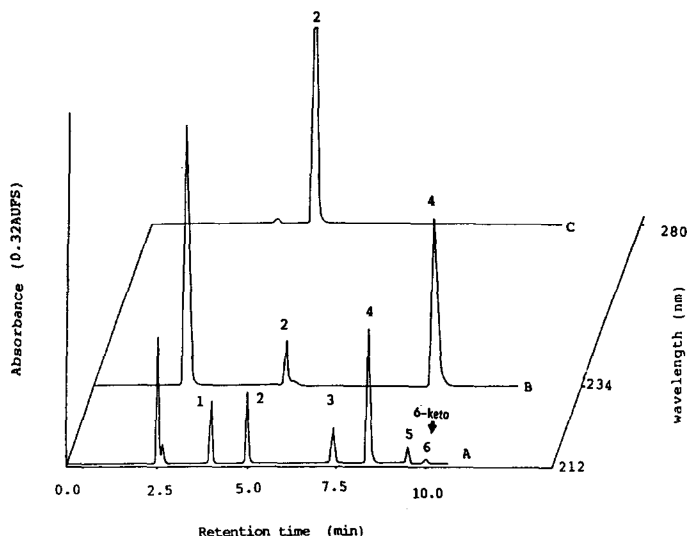

Fig. 1. HPLC of cholesterol and COPS standards on a cyano-bonded column with multiple wavelength scanning and UV detection. Solvent system, hexane–2-propanol (95:5, v/v); flow-rate, 1.0 ml/min. Peaks: (A) 1 = cholesterol, 2 = 4,6-dien-3-one, 3 = 25-OH, 4 = 7-keto, 5 = 7β-OH, 6 = 6-keto; (B) 2 = 4,6-dien-3-one, 4 = 7-keto; (C) 2 = 4,6-dien-3-one.

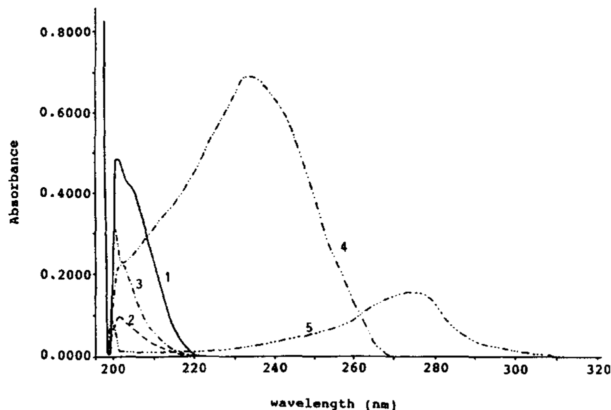

Fig. 2. UV spectra of cholesterol and COPS standards. 1 = Cholesterol; 2 = 7β-OH; 3 = 25-OH; 4 = 7-keto; 5 = 4,6-diene.

(Fig. 2). The selection of 212 nm rather than 205 nm for the detection of cholesterol, 7β-OH and 25-OH by HPLC is due to the fact that the latter was more readily subject to interference by the presence of solvents such as ethyl acetate, chloroform and 1,4-dioxane, which could lead to inaccurate quantification of COPS.

Fig. 3 shows the HPLC of cholesterol and eight COPS standards with RI detection. All compounds were adequately resolved within 17 min by employing the solvent system hexane–2-propanol (95:5, v/v) at a flow-rate at 1.0 ml/min. This solvent system was chosen based on a report by Nourooz-Zadeh [20], who used the same mobile phase at a flow-rate of 0.4 ml/min to separate cholesterol and eight COPS. However, baseline drift occurred during separation, which was accomplished within 60 min. By increasing the flow-rate to 1.0 ml/min it was possible to minimize the baseline drift and decrease the separation time to 17 min.

The separation efficiency of this method was determined by the capacity factors (k'); the k' values were cholesterol 1.51, 4,6-dien-3-one 2.05, 5,6α-EP 2.46, 5,6β-EP 2.63, 25-OH 2.76, 7-keto 3.01, 7β-OH 3.23 and triol 5.14. It is well established that the k' value should be between 2 and 10 to achieve an ideal separation [30]. However, in practice, eluting a sample within the k' range 1–20 is a more realistic goal [30]. Teng [28] used a silica gel-packed column to separate cholesterol and eleven COPS. Separation was achieved within 120 min. Obviously the drastically reduced separation time of COPS when using a cyano-bonded column is due to the interaction force between COPS and Si–$(CH_2)_3CN$ being smaller than that between COPS and Si–OH. In order to demonstrate the reproducibility of this method, a solution of COPS in hexane–2-propanol (95:5, v/v) was injected into the HPLC column five times. The R.S.D. with respect to the retention times of COPS was 0.00–1.00%.

The linearity responses of cholesterol and all COPS standards with UV detection at 212 nm or with RI detection on the cyano-bonded column were very high ($r^2 = 0.997$–0.999). The graph was obtained by plotting peak-area ratios against concentration ratios, and the peak-area ratios of cholesterol and COPS to the internal standard

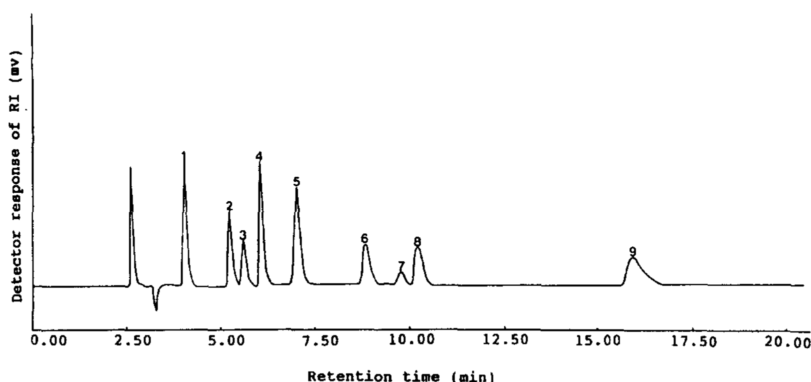

Fig. 3. HPLC of cholesterol and eight COPS standards on a cyano-bonded column with RI detection. Solvent system, hexane–2-propanol (95:5, v/v); flow-rate, 1.0 ml/min. Peaks: 1 = cholesterol; 2 = 4,6-dien-3-one; 3 = 5,6α-EP; 4 = 5,6β-EP; 5 = 25-OH; 6 = 7-keto; 7 = 7β-OH; 8 = 6-keto; 9 = triol.

were determined at four different concentration ratios of 0.5, 1.0, 1.5 and 2.0. However, the linearity response of 4,6-dien-3-one drastically decreased from 0.992 (r^2) to 0.747 (r^2) when the detection wavelength was changed from 234 to 280 nm. According to Beer's law, the absorption error can increase sharply with very dilute and very concentrated solutions. Hence the increased absorption of 4,6-dien-3-one at 280 nm could decrease the linearity response. 7-Keto with UV detection at 234 nm also showed the same phenomenon. To solve these problems, an appropriate range of concentrations (50–200 μg/ml) should be chosen so that high linearity (r^2 = 0.912) can be achieved.

Fig. 4 shows the HPLC of cholesterol and eight COPS standards detected at 212, 234 and 280 nm using the C_{18} column. A binary solvent system of acetonitrile–methanol (55:45, v/v) at a flow-rate of 0.5 ml/min was developed to resolve cholesterol and six COPS (4,6-dien-3-one, 3,5-diene, 25-OH, 7-keto, 7β-OH and 6-keto). Triol, 5,6α-EP and 5,6β-EP were not detected because of the absence of π electrons. However, 3,5-diene was the last eluted at 120 min. To decrease the retention time the flow-rate was maintained at 0.5 ml/min for the first 25 min and then increased linearly to 3.0 ml/min. The elution time of COPS could be reduced to within 60 min. Ansari and Smith [24] used a binary solvent system of acetonitrile–water (90:10, v/v) at a flow-rate of 1.0 ml/min to separate cholesterol and nine COPS. Although separation was accomplished within 60 min, baseline drift occurred and the resolution was poor. Also, cholesterol was eluted before 3,5-diene. This difference in elution order of cholesterol and 3,5-diene is due to their relative solubility in the solvent system used. 3,5-Diene had the strongest adsorption to the C_{18} packing material, so it was the last to be eluted. However, the separation time could be shortened to within 30 min if 3,5-diene was excluded from this experiment. As 3,5-diene is not commonly present in foods, its detection may be ignored because of its low cytoxicity [6]. Hence the application of a C_{18} column to separate common COPS in foods can still be adopted as a reference method.

Fig. 5 shows the HPLC of cholesterol and nine COPS standards on the C_{18} column with RI detection. All compounds were adequately resolved within 60 min using the same solvent system and flow-rate as described above. The k' values were triol 1.00, 25-OH 2.19, 7β-OH 2.59,

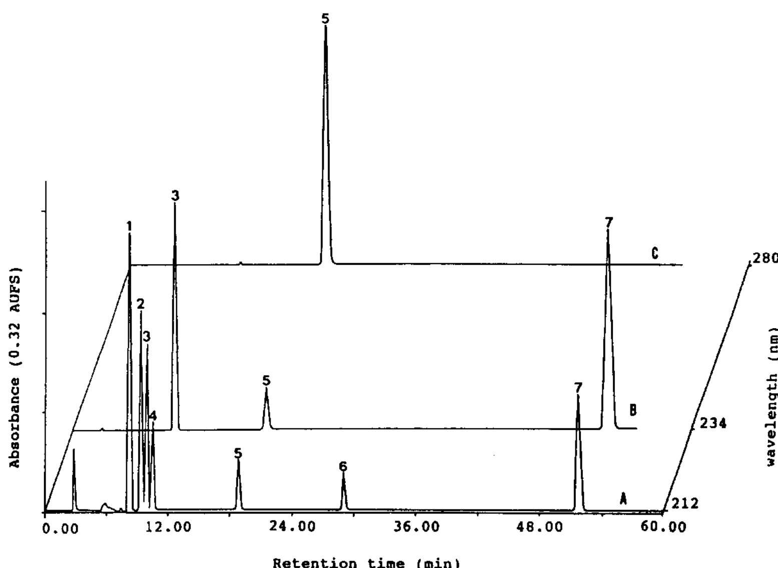

Fig. 4. HPLC of cholesterol and COPS standards on a C_{18} column with UV detection at 212, 234 and 280 nm. Solvent system acetonitrile–methanol (55:45, v/v); flow-rate, 0.5–3.0 ml/min. Peaks: (A) 1 = 25-OH, 2 = 7β-OH, 3 = 7-keto, 4 = 6-keto, 5 = 4,6-dien-3-one, 6 = cholesterol, 7 = 3,5-diene; (B) 3 = 7-keto, 5 = 4,6-dien-3-one, 7 = 3,5-diene; (C) 5 = 4,6-dien-3-one.

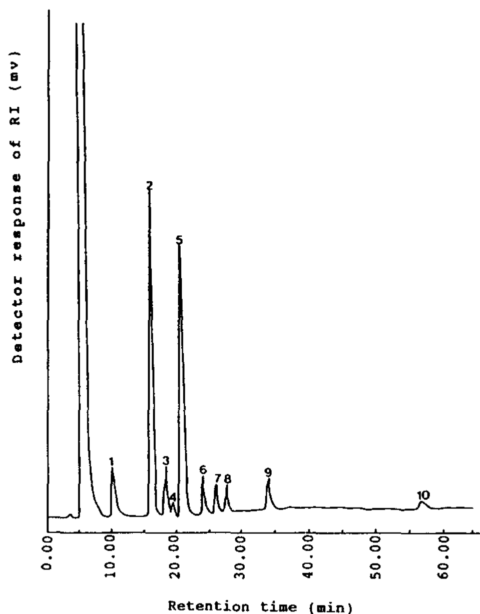

Fig. 5. HPLC of cholesterol and COPS standards on a C_{18} column with RI detection. Solvent system, acetonitrile–methanol (55:45, v/v); flow-rate, 0.5–3.0 ml/min. Peaks: 1 = triol; 2 = 25-OH; 3 = 7β-OH; 4 = 7-keto; 5 = 6-keto; 6 = 5,6α-EP; 7 = 5,6β-EP; 8 = 4,6-dien-3-one; 9 = cholesterol; 10 = 3,5-diene.

7-keto 2.79, 6-keto 3.12, 5,6α-EP 3.79, 5,6β-EP 4.19, 4,6-dien-3-one 4.59, cholesterol 5.78 and 3,5-diene 10.17. Compared with a normal-phase column, the elution time of COPS on the C_{18} column increased substantially from 17 to 60 min. However, a satisfactory separation could still be achieved because the k' values were in the range 1–11.

The linearity responses of cholesterol and standard COPS on the C_{18} column with UV detection at 212 nm and RI detection were very high ($r^2 = 0.980$–0.998). The r^2 values for 4,6-dien-3-one detected at 280 nm and for 7-keto and 3,5-diene detected at 234 nm were 0.950, 0.966 and 0.913, respectively. The linearity response of 4,6-dien-3-one on the C_{18} column with UV detection at 280 nm was much higher than that at 280 nm on the cyano-bonded column. This difference is due to the fact that the former was obtained by choosing a lower concentration range. On comparing the separation efficiency of the cyano-bonded column and C_{18} columns, the former was found to be superior in terms of retention time and resolution. Nevertheless, using this column to separate COPS has some drawbacks. First, 3,5-diene was eluted too fast and overlapped with the solvent peak. Second, the presence of residual triacylglycerol after extraction could form a large peak on the HPLC trace and thus interfere with the separation of cholesterol and some COPS. Also, UV detection of COPS was found to be 1000 times more sensitive than RI detection, the detection limits being 10 ng/g and 10 μg/g, respectively.

6-Keto was found to be a suitable internal standard because its structure is similar to that of other COPS, it did not interfere with the separation of the other compounds, it was completely eluted from the column and it was chemically inert towards other compounds. In addition, the linearity responses (r^2) for 6-keto on both columns with UV or RI detection was found to be between 0.992 and 0.999.

Extraction procedures

Table I gives the recoveries of cholesterol and COPS obtained by the three different extraction methods. Extraction by cold saponification and application of the Sep-Pak C_{18} cartridge were found to give higher recoveries than those obtained with the silica gel cartridge. Compared with the recoveries of the other COPS using cold saponification, triol was the lowest (86.9%), because hexane was used instead of diethyl ether to extract the COPS. The lower solubility of triol in hexane relative to diethyl ether resulted in a low recovery of triol. Hexane was adopted instead of diethyl ether because the latter has a strong odour and is volatile, which makes extraction difficult, and it can interfere with the UV detection of COPS. 3,5-Diene had the lowest recovery (95.4%) among the COPS extracted with the C_{18} cartridge. This is probably because a small portion of 3,5-diene was eluted together with triacylglycerol at the beginning by the ethyl acetate–methanol (1:1, v/v) solvent system used. It is also possible that a small portion of 3,5-diene has the strongest adsorption to the C_{18} packing material and thus its complete elution from the cartridge was not possible. Nevertheless, the recovery of 3,5-diene was still high in comparison with those of the other COPS ex-

TABLE I

RECOVERIES OF CHOLESTEROL AND COPS OBTAINED BY THREE DIFFERENT EXTRACTION METHODS

Means of quadruplicate analyses ± standard deviation.

Compound	Extraction method		
	Saponification[a]	C_{18}	Silica gel
3,5-Diene	102 ± 0.2^b	95.4 ± 0.5^c	50.2 ± 5.4^d
4,6-Dien-3-one	101 ± 1.2^b	100.8 ± 4.3^b	52.1 ± 3.3^c
Cholesterol	112 ± 3.2^b	111.6 ± 5.8^b	78.5 ± 1.6^c
5,6α-EP	96.8 ± 1.1^b	98.5 ± 2.4^b	82.3 ± 1.4^c
5,6β-EP	95.7 ± 3.2^b	96.7 ± 1.5^b	80.7 ± 0.2^c
25-OH	100 ± 0.3^b	99.4 ± 1.1^b	81.2 ± 3.9^c
7-Keto	108.9 ± 4.2^b	110.7 ± 2.8^b	99.7 ± 4.3^c
7β-OH	99.6 ± 2.2^b	101.2 ± 2.1^b	90.2 ± 2.8^c
6-Keto	101.1 ± 2.1^b	103.8 ± 2.5^b	95.2 ± 2.7^c
Triol	86.9 ± 3.1^b	97.7 ± 0.7^c	70.3 ± 3.1^d

[a] Conducted at room temperature for 18–20 h.
[b–d] Means within a row having different superscripts are significantly different ($P < 0.5$).

tracted with the silica gel cartridge. Most COPS had low recoveries when extracted with the silica gel cartridge, especially 3,5-diene and 4,6-dien-3-one (50.2 and 52.1%, respectively). This is probably due to the low polarity of 3,5-diene and 4,6-dien-3-one (which has the weakest adsorption to silica gel), and in turn this caused the elution of a small portion of both compounds together with triacylglycerol with the hexane solvent system used. For triol, the recovery is low (70.3%) because it has the strongest adsorption to silica gel and hence its complete elution from the cartridge was not possible. By comparison of the extraction efficiencies of the three methods, it was concluded that the application of a C_{18} cartridge and cold saponification was superior to a silica gel cartridge, and the use of a C_{18} cartridge was faster than cold saponification.

Determination of cholesterol oxides in heated lard

In order to demonstrate the extraction and separation efficiencies with the C_{18} cartridge and cyano-bonded column, lard heated at 180°C for 200 h was used as a reference sample. Fig. 6 shows the HPLC of COPS in this lard. Fig. 7 shows the HPLC of COPS in heated lard using co-chromatography with added standards. Only cholesterol and five COPS (4,6-dien-3-one, 5,6α-EP, 7-keto, 7β-OH and triol) were identified. No 6-keto was found in fresh lard. 6-Keto was present in heated lard is because it was added as an internal standard. The quantitative changes in COPS during heating of lard have been described in another paper [29].

In conclusion, the extraction and separation of

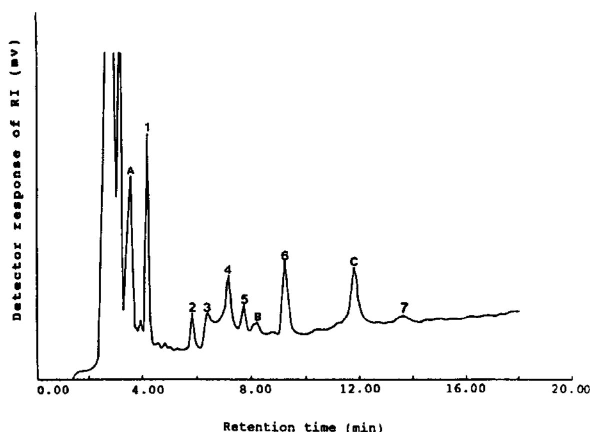

Fig. 6. HPLC of COPS in lard heated at 180°C for 200 h with RI detection. Solvent system, hexane–2-propanol (95:5, v/v); flow-rate, 1.0 ml/min. Peaks: 1 = cholesterol; 2 = 4,6-dien-3-one; 3 = 5,6α-EP (5,6β-EP); 4 = 7-keto; 5 = 7β-OH; 6 = 6-keto; 7 = triol; A,B,C = unknowns.

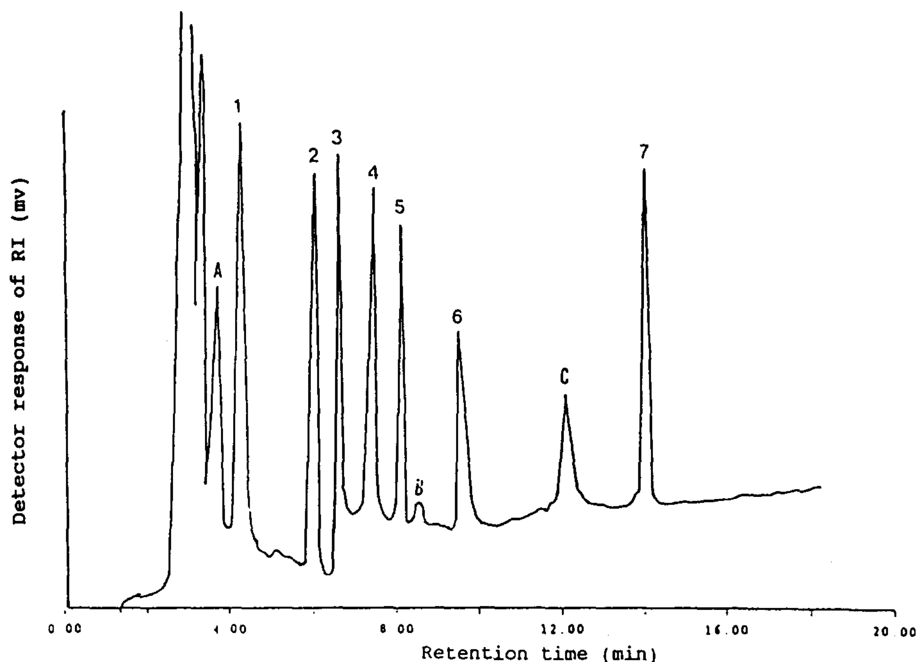

Fig. 7. HPLC of COPS in heated lard by co-chromatography with added standards with RI detection. Solvent system, hexane–2-propanol (95:5, v/v); flow-rate, 1.0 ml/min. Peaks: 1 = cholesterol; 2 = 4,6-dien-3-one; 3 = 5,6α-EP (5,6β-EP); 4 = 7-keto; 5 = 7β-OH; 6 = 6-keto; 7 = triol; A,B,C = unknowns.

COPS using a Sep-Pak C_{18} cartridge and a cyano-bonded column with UV detection were found to be the most efficient in terms of retention time and sensitivity. Further research is necessary to determine what unknown COPS may present in heated lard.

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REFERENCES

1 L.L. Smith, *Cholesterol Autoxidation*, Plenum Press, New York, 1981.
2 S.K. Peng, P. Tham, C.B. Taylor and B. Mikkelson, *Am. J. Clin. Nutr.*, 32 (1979) 1033.
3 C.B. Taylor, S.K. Peng, N.T. Werthessen, P. Tham and K.T. Lee, *Am. J. Clin. Nutr.*, 32 (1979) 40.
4 H. Imai, N.T. Werthessen, V. Subramanyam, P.W. Lequesne, A.H. Soloway and M. Kanisawa, *Science*, 207 (1980) 651.
5 G.A.S. Ansari, R.D. Walker, V.B. Smart and L.L. Smith, *Food Chem. Toxicol.*, 20 (1982) 35.
6 E.T. Finocchiaro and T. Richardson, *J. Food Protect.*, 46 (1983) 917.
7 S.W. Park and P.B. Addis, *J. Agric. Food Chem.*, 34 (1986a) 653.
8 S.W. Park and P.B. Addis, *J. Food Sci.*, 51 (1986) 1380.
9 S.W. Park and P.B. Addis, *J. Food Sci.*, 52 (1987) 1500.
10 B.D. Sander, P.B. Addis, S.W. Park and D.E. Smith, *J. Food Protect.*, 552 (1989) 109.
11 B.D. Sander, D.E. Smith, P.B. Addis and S.W. Park, *J. Food Sci.*, 54 (1989) 874.
12 J.E. Pie, K. Spahis and C. Seillan, *J. Agric. Food Chem.*, 38 (1990) 973.
13 P.S. Yan and P.J. White, *J. Am. Oil Chem. Soc.*, 67 (1990) 927.
14 G. Maerker and J. Unruh, *J. Am. Oil Chem. Soc.*, 63 (1986) 767.
15 L.S. Tsai and C.A. Hudson, *J. Food Sci.*, 49 (1984) 1245.
16 L.S. Tsai and C.A. Hudson, *J. Food Sci.*, 50 (1985) 229, 237.
17 I.L. Kou and R.S. Holmes, *J. Chromatogr.*, 330 (1985) 339.
18 S.R. Missler, B.A. Wasilchuk and C. Merritt, *J. Food Sci.*, 50 (1985) 595.
19 S.W. Park and P.B. Addis, *J. Food Sci.*, 50 (1985) 1437.
20 J. Nourooz-Zadeh, *J. Agric. Food Chem.*, 38 (1990) 1667.

21 W.W. Nawar, S.K. Kim and M. Vajdi, *J. Am. Oil Chem. Soc.*, 68 (1991) 496.
22 M.P. Zubillaga and G. Maerker, *J. Food Sci.*, 56 (1991) 1194.
23 J.I. Teng, M.J. Kulig, L.L. Smith, G. Kan and E.V. Lier, *J. Org. Chem.*, 38 (1973) 119.
24 G.A.S. Ansari and L.L. Smith, *J. Chromatogr.*, 175 (1979) 307.
25 E.T. Finocchiaro, K. Lee and T. Richardson, *J. Am. Oil Chem. Soc.*, 61 (1984) 877.
26 S.W. Park and P.B. Addis, *Anal. Biochem.*, 52 (1985b) 275.
27 L.S. Tsai and C.A. Hudson, *J. Am. Oil Chem. Soc.*, 58 (1981) 931.
28 J.I. Teng, *LC·GC*, 9 (1991) 214.
29 Y.C. Chen, C.P. Chiu and B.H. Chen, *Food Chem.*, in press.
30 J.W. Dolan, *LC·GC*, 9 (1991) 466.
31 *SAS/STAT Guide for Personal Computers, Version 6ed*, SAS Instruments, Cary, NC, 1985.