Rapid Analysis of Oxidized Cholesterol Derivatives by High-Performance Liquid Chromatography Combined with Diode-Array Ultraviolet and Evaporative Laser Light-Scattering Detection

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ABSTRACT: Extensive evidence of the deleterious biological effects of oxidized 5-cholesten-3β-ol (cholesterol) derivatives has led to great interest in their detection. We observed that known oxidized cholesterol derivatives can be rapidly quantitated by combining reversed-phase high-performance liquid chromatography (HPLC) with ultraviolet (UV) absorption and evaporative laser light-scattering (ELSD) detection. Using a 20 × 0.46 cm C18 HPLC column and methanol/acetonitrile (60:40, vol/vol) as the mobile phase at 1.0 mL/min, 10 species of oxidized cholesterol derivative were measured by UV (205, 234, and 280 nm) while 5-cholestan-5α,6α-epoxy-3β-ol (5α-epoxycholesterol), 5-cholestan-5β,6β-epoxy-3β-ol (5β-epoxycholesterol), and 5-cholestan-3β,5α,6β-triol (cholestanetriol) were detected by only ELSD. The minimal limits of detection ranged from 100 to 500 ng depending on sterol and detector. The response was linear in the range 0–1000 or 0–2000 ng depending on detector. These oxidized cholesterol derivatives were also identified by HPLC/mass spectrometry analysis combined with UV detector. Heated tallow contained cholestanetriol, 5-cholesten-3β,7α-diol (7α-hydroxycholesterol), 5-cholesten-3β,7β-diol (7β-hydroxycholesterol), 5-cholesten-3β-ol-7-one (7-ketocholesterol), 5α- and 5β-epoxycholesterols under the developed analysis condition. Photooxidized cholesterol had cholestanetriol, 7α- and 7β-hydroxycholesterols and 3,5-cholestadien-7-one. On the other hand, 7α- and 7β-hydroxycholesterols, 7-ketocholesterol, 5α- and 5β-epoxycholesterols and 3,5-cholestadien-7-one were observed in copper-oxidized low-density lipoprotein. Thus, this developed HPLC analysis method could be applied to identification of oxidized cholesterol derivatives in food and biological specimen.

Paper no. J8986 in *JAOCS 76,* 863–871 (July 1999).

KEY WORDS: Cholesterol, ELSD, HPLC, LDL, oxidized cholesterol, photooxidation, tallow, UV.

Lipid oxidation products are present in unknown levels in the processed foods and human tissues. These oxidized lipids show deleterious activities such as atherogenic action throughout the initiation of endothelial injury, the accumulation of plaque, and termination phase of thrombosis (1). Among oxidized lipids, oxidized 5-cholesten-3β-ol (cholesterol) derivatives display specific diverse biological activities such as cytotoxicity, carcinogenicity, atherogenicity, modulation of lipid metabolism, and suppression of immune function (2). Convincing evidence of the deleterious biological effects led to development of various methods for the analysis of oxidized cholesterol derivatives based on gas chromatography (GC) and high-performance liquid chromatography (HPLC), and mass spectrometry (MS) (3,4). The most successful of these have been a combination of GC and online mass spectrometry (GC/MS). These methods, however, require expensive equipment not routinely available. In contrast to GC analysis of oxidized cholesterol derivatives, the HPLC analysis condition of oxidized cholesterol derivatives is not completely established. Several studies measured the levels of oxidized cholesterol derivatives by HPLC with ultraviolet (UV) (5–8), refractive index (RI) (9), evaporative light-scattering detection (ELSD) (10–12), and flame-ionization detector (FID) (13), using various adsorbent columns. Many of these analytical methods leave unresolved problems concerning sensitivity, baseline instability, and in application to samples derived from foods and biological specimen; however, Caboni *et al.* (12) showed superior ELSD detection of oxidized cholesterol derivatives using cyano-bonded normal-phase column within 30 min. We also wish to report the development of a rapid and inexpensive method for the analysis of common oxidized cholesterol derivatives, and its application in the identification of oxidized cholesterol derivatives in food, plasma lipoproteins, and in photooxidized materials using reversed-phase column.

EXPERIMENTAL PROCEDURES

Chemicals. Cholesterol and its oxidized derivative standards were purchased from Sigma Chemical Co. (St. Louis, MO), Steraloids Inc. (Wilton, NH), and Research Plus (Bayonne, NJ), shown in Table 1. Commercially deodorized beef tallow was obtained from Wako Pure Chemicals (Osaka, Japan). Other solvents and chemicals were of reagent grade or better quality and purchased from a local supplier (Caledon Chemicals, Toronto, Canada).

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TABLE 1
Peak Identification[a]

Peak no.	Compound	Abbreviation
1	25-Hydroxycholesterol	25HOC[a]
2	26-Hydroxycholesterol	26HOC[b]
3	Cholestanetriol	Ctriol[a]
4	7α-Hydroxycholesterol	7αHOC[c]
5	7β-Hydroxycholesterol	7βHOC[a]
6	7-Ketocholesterol	7ketoC[a]
7	5β-Epoxycholesterol	5βEpoxyC[c]
8	5α-Epoxycholesterol	5αEpoxyC[a]
9	4,6-Cholestadien-3-one	46CD[c]
10	3,5-Cholestadien-7-one	35CD[c]
11	Cholesterol	C[a]
12	20α-Hydroxycholesterol	20αOC[a]
13	6-Ketocholestanol	6KetoC[a]
14	19-Hydroxycholesterol	19HOC[a]
15	5α-Cholestan-3,6-dione	5C36D[c]

[a]Sources: (a) Sigma Chemical Co. (St. Louis, MO), (b) Research Plus (Bayonne, NJ), (c) Steraloid, Inc. (Wilton, NH).

Instruments. The HPLC instrument used for oxidized cholesterol derivatives had two HPLC pumps (Waters Model 510; Waters, Bedford, MA), manual injector (Waters Model U6K), and a system interface module for computerized peak integration and data handling (Waters Maxima 820 chromatography Workstation). Two detectors were used for this analysis of oxidized cholesterol derivatives. The UV absorbance was determined with a photodiode-array system (Waters Model 990) consisting of a 512 diode UV/Visible detector and a stand-alone NEC personal computer. The other was an ELSD system, consisting of a VAREX ELSD II (Varex, Baitonsville, MD) equipped with a HP 3396A integrator (Hewlett-Packard, Palo Alto, CA).

HPLC column. Cholesterol and its oxidative derivatives were separated on a Supelcosil LC-18 column (25 cm × 4.6 cm i.d., particle size: 5 μm) purchased from Supelco, Inc. (Bellefonte, PA).

Analysis using photodiode-array detection. Twenty-five μL (0.004–1 mg/mL) of each available oxidized cholesterol derivative standard were injected into the HPLC analysis system by manual injection after they were dissolved in dichloromethane. The mobile phase for separation was a mixture of methanol and acetonitrile (60:40, vol/vol), and lasted for 30 min; the solvent mixture was maintained at a flow rate of 1.0 mL/min. UV detection was performed simultaneously at different wavelengths between 190 and 290 nm.

Detection of oxidized cholesterol derivatives by ELSD. Simultaneous HPLC analysis of oxidized cholesterol derivatives was performed after UV analysis by connection of the diode-array and ELSD detectors. Evaporative temperature of ELSD was set at 110°C and a stream of nitrogen gas at a flow rate of 40 mL/min.

MS analysis. Chromatographic analysis of oxidized cholesterol derivatives was also performed on a reversed-phase column using a Hewlett-Packard Model 1050 Liquid Chromatograph connected to a Hewlett-Packard Model 5988B Quadrupole mass spectrometer equipped with nebulizer assisted electrospray ionization (ESI) interface for the accurate identification of each oxidized cholesterol derivative. This analysis was simultaneously performed by post-column method after UV detection by connection of photodiode-array detector and mass spectrometer. After passing through photodiode-array detector, oxidized cholesterol derivative standards mixture was subjected to HPLC/ESI/MS analysis. The column was eluted with the mixture of methanol and acetonitrile (60:40, vol/vol) at a flow rate of 0.8 mL/min; the column eluant was mixed with the mobile phase containing 0.5% ammonium hydroxide at a post-column mixing joint. The mobile phase containing ammonium hydroxide pumped at 0.3 mL/min with other HPLC pump. Positive ionization spectra were taken in the *m/z* range 300–500. Selected-ion chromatograms mass was retrieved from the HPLC/ESI/MS data.

Isolation of oxidized cholesterol derivatives from heated tallow. Beef tallow (1 g) dissolved in 5 mL chloroform was placed in glass dish (diameter: 10 cm). The solvent was evaporated under nitrogen gas to make a thin oil film and then heated for 8 h at 80°C in an electric oven. The heated sample was saponified by 0.5 M KOH in ethanol containing 0.001% butylated hydroxytoluene at 4°C for 24 h to avoid further autooxidation (14). Saponified sample was applied on a silicic acid column (silica gel 60, 70–230 mesh; E. Merck, Darmstadt, Germany, 30 cm × 24 mm) and fractionated by successive elution with 30 mL *n*-hexane, 15 mL diethyl ether, and finally 25 mL methanol at 4°C in dark room. An aliquot eluted by methanol was dried in a rotary evaporator and finally *in vacuo*.

Photooxidation of cholesterol. Photooxidized cholesterol was prepared by the modified photosensitized oxidation method described by Neff *et al*. (15). Cholesterol (1 mg) was dissolved into 5 mL of 0.1 mM methylene blue in chloroform/methanol (1:1, vol/vol) in a 15-mL test tube on ice bath, which was placed under a 500 W photographers lamp for 5 h. After reaction, sterol was purified using a Florisil column to remove methylene blue.

Preparation of copper-oxidized low-density lipoprotein (LDL). Native LDL was isolated from normal human plasma by sequential ultracentrifugation (16). LDL was dialyzed for 24 h at 4°C in the dark against vacuum-degassed 0.05 M potassium phosphate buffer (pH 7.4). The dialyzed LDL solution (1 mg protein/mL) was transferred into a dialysis bag and immersed in a 100-fold volume of 0.05 M potassium phosphate buffer (pH 7.4) with 5 μM $CuSO_4$ for oxidation. The oxidation system was kept in the dark at room temperature, and oxygen was bubbled continuously through the external buffer. After incubation for 24 h, lipids were extracted immediately from oxidized LDL using chloroform/methanol (2:1, vol/vol) containing 0.001% butylated hydroxytoluene by the method of Folch *et al*. (17). Extracted lipids were saponified the same as in heated tallow.

RESULTS

HPLC separation and detection of oxidized cholesterol derivatives. Firstly, UV absorption spectra were measured and recorded for each (purity, 95–99%) oxidized cholesterol de-

rivative standard. Figure 1 shows UV absorbance for cholesterol and eight available species of oxidized cholesterol derivative. Maximal absorption of 5-cholesten-3β,7α-diol (7α-hydroxycholesterol), 5-cholesten-3β,7β-diol (7β-hydroxy-cholesterol), 5-cholesten-3β,20α-diol (20α-hydroxycholesterol), 5-cholesten-3β,25-diol (25-hydroxycholesterol), 5-cholesten-3β,26-diol (26-hydroxycholesterol), and cholesterol was 205 nm. 5-Cholestan-3β-ol-6-one (6-ketocholestanol), 5-cholesten-3β,19-diol (19-hydroxycholesterol), and 5α-cholestan-3,6-dien also had maximal absorption at 205 nm (data not shown). Maximal absorption of 5-cholesten-3β-ol-7-one (7-ketocholesterol) was at 234 nm. In addition, both 4,6-cholestadien-3-one and 3,5-cholestadien-7-one had the maximal absorption at 280 nm. However, no UV absorption was observed in 5-cholestan-5α,6α-epoxy-3β-ol (5α-epoxycholesterol), 5-cholestan-5β,6β-epoxy-3β-ol (5β-epoxycholesterol), and 5-cholestan-3β,5α,6β-triol (cholestanetriol).

Figure 2 shows the HPLC for cholesterol and its oxidative derivatives using a reversed-phase column with UV detection at 205, 234, and 280 nm and ELSD, respectively. It was possible to separate 10 species of oxidized cholesterol derivatives and cholesterol within 30 min. The maximal absorbance of each oxidized cholesterol derivative showed typical absorption as shown in Figure 1; 7α- (Peak 4), 7β- (Peak 5), 25- (Peak 1) and 26- (Peak 2) hydroxycholesterols and cholesterol

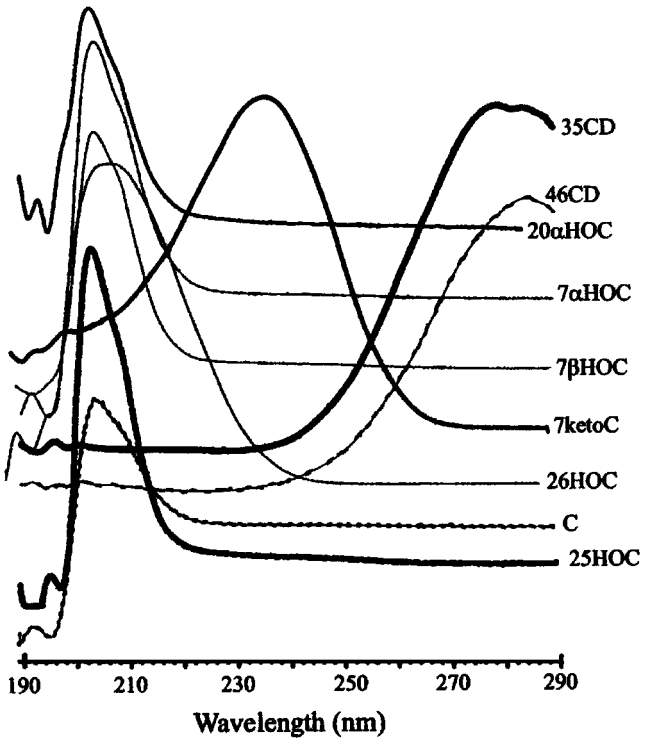

FIG. 1. Ultraviolet (UV) spectra of cholesterol and oxidized cholesterol derivative standards. 35CD, 3,5-cholestadien-7-one; 46CD,4,6-cholestadien-3-one; 20αHOC, 20α hydroxycholesterol; 7αHOC, 7α-hydroxycholesterol; 7βHOC, 7β-hydroxycholesterol; 7KetoC, 7-ketocholestanol; 26HOC, 26-hydroxycholesterol; C, cholesterol; 25HOC, 25-hydroxycholesterol.

(Peak 11) were detected at only 205 nm. 7-Ketocholesterol (Peak 6) demonstrated two specific absorbances at both 205 and 234 nm; however, detection at 234 nm gave a higher response. On the other hand, it was found that both 3,5-cholestadien-7-one (Peak 10) and 4,6-cholestadien-3-one (Peak 9) had the highest responses at 280 nm, although these oxidative derivatives were also detected at 205 and 234 nm. Moreover, 19- and 20α-hydroxycholesterols, 6-ketocholestanol, and 5α-cholestan-3,6-dione were also separated at only 205 nm; however, these were not resolved because their retention times were the same as in other oxidized cholesterol derivatives (19-hydroxycholesterol/7-ketocholesterol, 20α-hydroxycholesterol/25-hydroxycholesterol, 6-ketocholestanol/7α-hydroxycholesterol, 5α-cholestan-3,6-dione/5β-epoxycholesterol). Although cholestanetriol, 5α- and 5β-epoxycholesterols were adequately resolved from other compounds, they were effectively detected only by ELSD. The retention time of 5β-epoxycholesterol was the same as that of 5α-cholestan-3,6-dione.

Linearity of UV and ELSD responses. Figure 3 shows the calibration curves for HPLC-UV and -ELSD responses of cholesterol and 10 species of oxidized cholesterol derivative in the range 0–2000 or 0–1000 ng. These results show that good linearity was obtained for HPLC–UV and –ELSD of 7α-, 7β-, 20α-, 25- and 26-hydroxycholesterols, 7-ketocholesterol, 3,5-cholestadien-7-one, 4,6-cholestadien-3-one, 5α- and 5β-epoxycholesterols and cholestanetriol (5α- and 5β-epoxycholesterols and cholestanetriol were only in ELSD). The minimal limits of detection of each sterols varied from 100 to 500 ng [100 ng: 5α- and 5β-epoxycholesterols, 7-ketocholesterol, 3,5-cholestadien-7-one, 4,6-cholestadien-3-one; 200 ng: 7α-, 7β-, 20α-, 25- and 26-hydroxycholesterols, 400 ng: cholestanetriol; 500 ng: cholesterol], depending on the compound and the detector. The correlation coefficient for cholesterol and oxidized cholesterol derivatives was between 0.996 and 0.999 (cholesterol, 0.998; 7-ketocholesterol, 0.996; 7α-hydroxycholesterol, 0.999, 7β-hydroxycholesterol, 0.997; 4,6-cholestadien-3-one, 0.999; 3,5-cholestadien-7-one, 0.997; 25-hydroxycholesterol, 0.998; 26-hydroxycholesterol, 0.999) in UV responses and between 0.981 and 0.997 (cholesterol, 0.995; 7-ketocholesterol, 0.994; 7α-hydroxycholesterol, 0.995, 7β-hydroxycholesterol, 0.988; 4,6-cholestadien-3-one, 0.997; 3,5-cholestadien-7-one, 0.994; 25-hydroxycholesterol, 0.981; 26-hydroxycholesterol, 0.991; 5α-epoxycholesterol, 0.993; 5β-epoxycholesterol, 0.997; cholestanetriol, 0.996) in ELSD, respectively.

Determination of oxidized cholesterol derivatives in heated tallow. The tallow heated at 80°C for 8 h was first applied to the present HPLC analysis system as a reference sample to demonstrate the application and separation efficiencies with reversed-phase column. Figure 4 shows the HPLC pattern of oxidized cholesterol derivatives in heated tallow by UV and ELSD detections. Oxidized cholesterol derivatives were not found in fresh tallow; however, heated tallow had cholestanetriol, 7α- and 7β-hydroxycholesterols, 5α- and 5β-epoxycholesterols, and 7-ketocholesterol, although there are

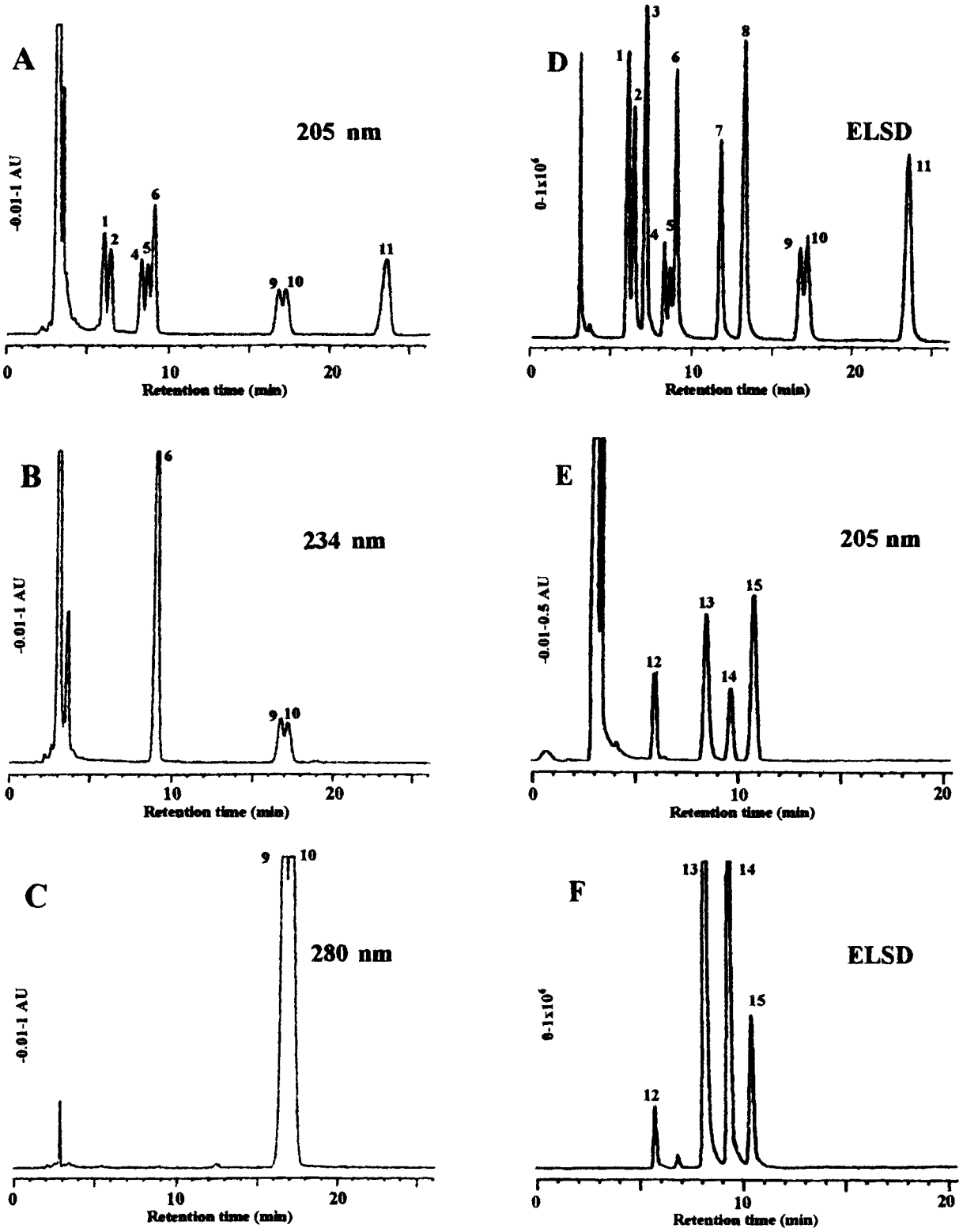

FIG. 2. High-performance liquid chromatography (HPLC) of cholesterol and oxidized cholesterol derivative standards on a reversed-phase column with diode-array UV and evaporative light-scattering detector (ELSD) detection. Peak number was the same as in Table 1. The mixture of 7α-, 7β-, 25- and 26-hydroxycholesterols, 5α- and 5β-epoxycholesterols, cholestanetriol, 7-ketocholesterol, 3,5-cholestadien-7-one and 4,6-cholestadien-3-one was injected in panels A, B, C, and D. Panels E and F show the chromatograms of the mixture of 19- and 20α-hydroxycholesterols, 6-keto-cholestanol and 5α-cholestan-3,6-dione. See Figure 1 for other abbreviations.

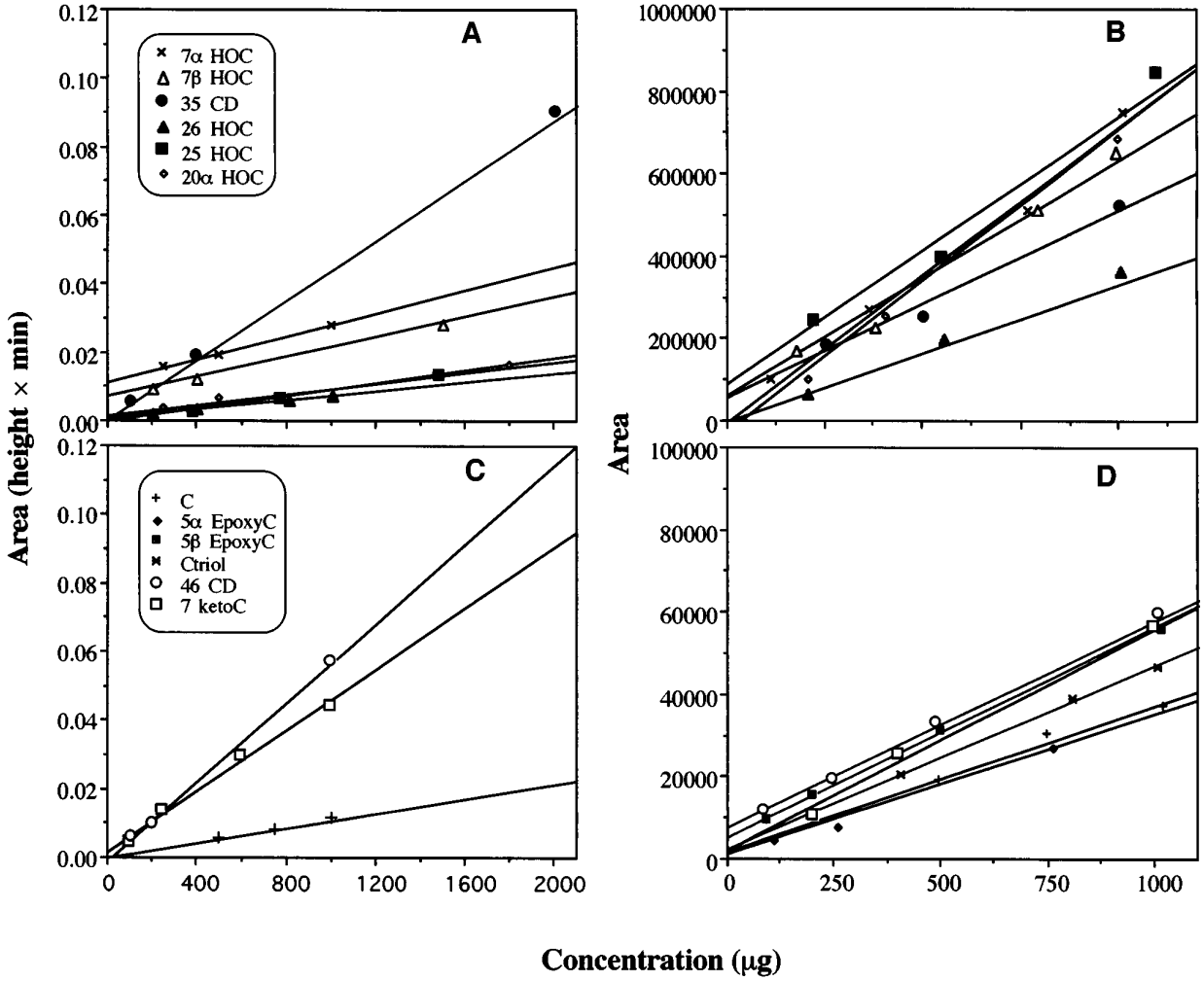

FIG. 3. Calibration graphs for HPLC of cholesterol and its oxidative derivatives using diode-array UV and ELSD detection. Each point represents mean of triplicate measurement. See Figures 1 and 2 for abbreviations. (A) UV (1); (B) ELSD (1); (C) UV (2); (D) ELSD (2).

some unidentified peaks in heated tallow as observed in Figure 4. The quantitative data are summarized in Table 2 along with selected values from the literature (18,19).

Determination of oxidized cholesterol derivatives in photooxidized cholesterol. Irradiation of cholesterol in the presence of methylene blue produced various oxidized cholesterol derivatives such as cholestanetriol, 7α- and 7β-hydroxycholesterols, 5α- and 5β-epoxycholesterols, 7-ketocholesterol and 3,5-cholestadien-7-one; however, pure cholesterol used in this reaction had no oxidized cholesterol derivatives before photooxidation. Unknown oxidized cholesterol derivatives were also found in this sample. These may be cholesterol hydroperoxides because we found the red spot specific for hydroperoxide by spraying of *N,N*-dimethyl-*p*-phenylene-diamine solution after the photooxidized sample was developed in thin-layer chromatography. The quantitative data are summarized in Table 3 along with selected values from the literature (20,21).

Determination of oxidized cholesterol derivatives in copper-oxidized LDL. Oxidized cholesterol derivatives were not observed in native LDL; however, copper-oxidized LDL had 7α- and 7β-hydroxycholesterols, 5α- and 5β-epoxycholesterols, 7-ketocholesterol, and 3,5-cholestadien-7-one. The quantitative data are summarized in Table 4 along with selected values from the literature (22,23).

Identification of major oxidized cholesterol derivatives by HPLC/ESI/MS. Figure 5 shows the total ion current profile and mass spectra of major oxidized cholesterol derivatives. The characteristic ion of each oxidized cholesterol derivatives was as follows: 25-hydroxycholesterol, 367, 385, 413, 425, and 441; 26-hydroxycholesterol, 367, 385, 413, 425, and 441; cholestanetriol, 367, 385, 413, 438, and 441; 7α-hydroxycholesterol, 367, 385, 413, 425, and 441; 7β-hydroxycholesterol, 367, 385, 413, 425, and 441; 7-ketocholesterol, 401 and 439; 5α-epoxycholesterol, 367, 385, 413, 425, and 441; 5β-epoxycholesterol, 367, 385, 413, 425, and 441; 3,5-cholestadiene-7-one, 383, 405, and 421; and 4,6-cholestadiene-3-one, 383, 405, and 421.

DISCUSSION

The present study showed the development of rapid analysis

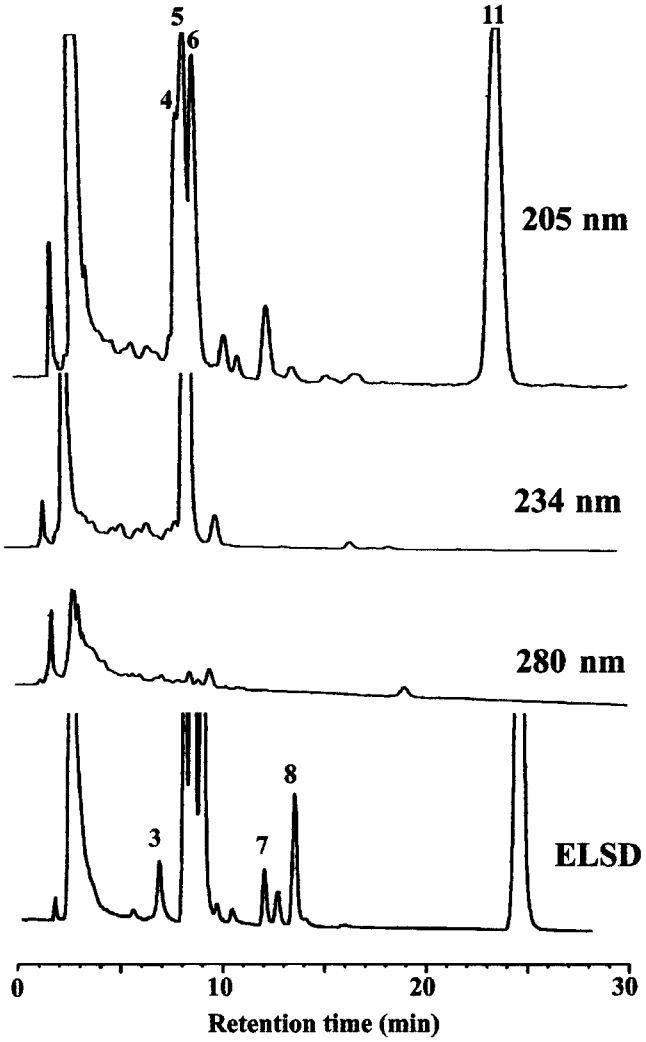

FIG. 4. HPLC of oxidized cholesterol derivatives in tallow heated at 80°C for 8 h. Tallow was heated at 80°C for 8 h in electric oven. Sample was injected on column after saponification by 0.5 M KOH in ethanol at 4°C and concentrated using silica gel column. See Figure 2 for abbreviations.

TABLE 2
Content of Oxidized Cholesterol Derivatives in Heated Tallow[a]

	Heated tallow		
Oxidized cholesterols	A (μg/g)	B (%)	C (ppm)
Ctriol	7.7 ± 1.0	n.d.	90
7αHOC	27.9 ± 3.9	3	40
7βHOC	98.9 ± 7.7	2.2	40
7ketoC	55.1 ± 7.5	10	10
5αEpoxyC	8.2 ± 2.5	4	15
5βEpoxyC	12.9 ± 4.6	n.d.	
35CD	n.d.	n.d.	n.d.

[a]A: Tallow was heated as described in text, values are mean ± SE of three different data. B: Tallow was heated at 155°C for 376 h. Values are the average of triplicate analysis (18). C: Tallow was heated for 60 h under deep frying conditions. Values are the average of 2–7 samples (19). See Table 1 for abbreviations.

TABLE 3
Content of Oxidized Cholesterol Derivatives in Photooxidized Cholesterol[a]

	Photooxidized cholesterol		
Oxidized cholesterols	A (μg/mg)	B (mg/g)	C (mg/g)
Ctriol	0.9 ± 0.4	n.d.	62
7αHOC	20.7 ± 4.9	0.8	n.d..
7βHOC	11.0 ± 1.5	0.5	507
7ketoC	16.9 ± 2.2	n.d..	200
5αEpoxyC	1.8 ± 0.3	n.d..	2522
5βEpoxyC	2.2 ± 0.5	n.d..	n.d..
35CD	0.1 ± 0.0	n.d..	n.d..

[a]A: Cholesterol was photooxidized as described in text, values are mean ± SE of three different data. B: Eggnog mix was exposed to fluorescent light for prolonged periods up to 80 d. Values are the single data (20). C: Dried egg-yolk powder was irradiated with ultraviolet light for 3 wk after storage. Values are the average of duplicate analysis (21). See Table 1 for abbreviations.

TABLE 4
Content of Oxidized Cholesterol Derivatives in Copper-Oxidized LDL[a]

	Copper-Oxidized LDL		
Oxidized cholesterols	A (μg/mL)	B (μg/g)	C (μg/mL)
Ctriol	n.d.	0.214	0.64
7αHOC	356.3 ± 44.7	19.65	12.7
7βHOC	189.6 ± 7.5	22.89	14.1
7ketoC	96.8 ± 22.2	32.3	37.8
5αEpoxyC	57.6 ± 10.5	2.855	4.4
5βEpoxyC	9.9 ± 0.7	12.83	17.8
35CD	10.4 ± 1.5	n.d.	n.d.

[a]A: Low-density lipoprotein (LDL) was oxidized as described in text, values are mean ± SE of three different data. B: LDL was incubated into 100 mM phosphate-buffered (pH 7.4) saline containing 20 μM $CuSO_4$. Values are the average of duplicate analysis (22). C: LDL was incubated with 5 μM $CuSO_4$ in 10 mM potassium phosphate buffered saline at pH 7.4 and 37°C for 24 h. Values are the average of duplicate analysis (23). See Table 1 for abbreviations.

method of oxidized cholesterol derivatives. In an earlier stage of the development of HPLC analysis for oxidized cholesterol derivatives, Tsai and Hudson (5) reported the good resolution of oxidized cholesterol derivatives using normal-phase column combined with UV and differential refractometer. However, this method partly gave poor resolution of some oxidized cholesterol derivatives and failed to elute cholestanetriol. Contrary to this result, Maerker *et al*. (13) observed that normal-phase gradient HPLC with FID detection was an effective procedure for separation and quantitation of oxidized cholesterol derivatives; however, HPLC–FID analysis is not commonly applied compared to UV or ELSD analysis.

Firstly, UV absorption for 7α- 7β- and 25-hydroxycholesterols, cholesterol, and 7-ketocholesterol was consistent with the report of Csallany *et al*. (8). Recently, Chen and Chen (9) reported superior resolution of oxidized cholesterol derivatives by HPLC combined with UV and RI using cyano-bonded or reversed-phase column. In fact, this method was applied to analysis of heated lard; however, RI response was not as sensitive as UV response. On the other hand, Kermasha *et al*. (10) and Lakritz and Jones (11) also showed superior resolution of oxidized cholesterol derivatives using HPLC with ELSD. In the method of Kermasha, eight species of oxi-

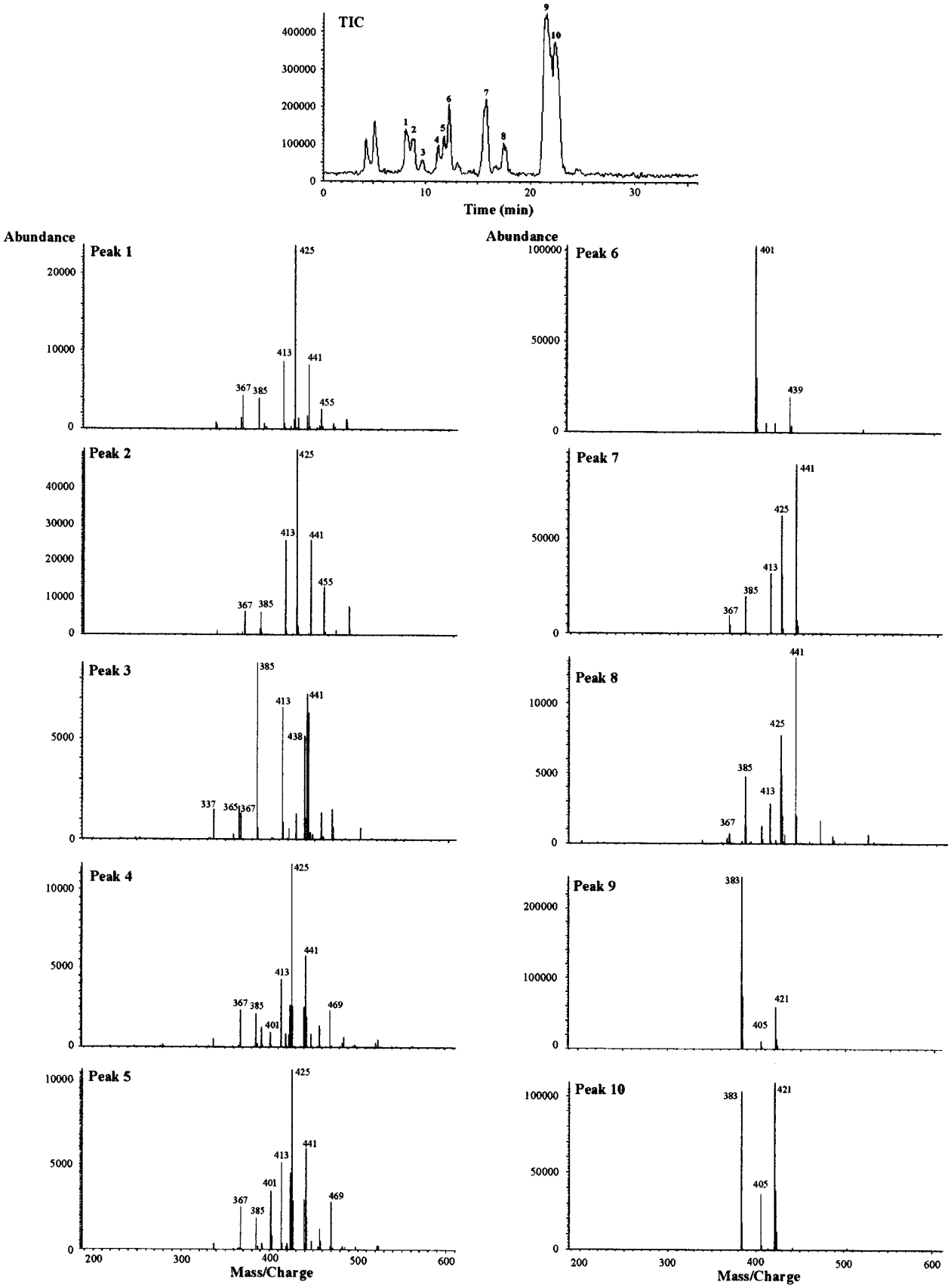

FIG. 5. Reversed-phase high-performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI/MS) of oxidized cholesterol derivative standards mixture. Peak number was the same as in Table 1. Total positive ion current; *m/z* 300–500. HPLC/ESI/MS equipment and operating conditions are shown in the Experimental Procedures section.

dized cholesterol derivative were separated by normal-phase gradient analysis, and the response was linear in the range 0–500 μg/mL, although the baseline of UV was not stable and elution time was very long (50 min) in this method compared to our condition. Recently, Lakritz and Jones (11) found excellent analysis of oxidized cholesterol derivatives using the specific 16% alumina/84% silica column. However, these two methods were not applied to foods and biological specimen. Contrary to these results, cholesterol and 10 species of oxidized cholesterol derivative containing polar compounds such as cholestanetriol were adequately separated within 30 min in our method. Baseline was also stable and response was highly sensitive. Caboni *et al*. (12) also detected 11 species of oxidized cholesterol derivatives containing hydroperoxycholesterols below 700 ng, although the retention time of 5β-epoxycholesterol was the same as in 4β-hydroxycholesterol in their analytical condition. In addition, our analytical method was suitable for the identification of oxidized cholesterol derivatives in food, plasma lipoproteins, and photooxidized cholesterol films. We tried to use 6-ketocholestanol, 19-hydroxycholesterol, and 5α-cholestan-3,6-dione as internal standards; however, their retention times were similar to those of other compounds. Therefore, further studies on this observation are required to confirm more useful calculating method using internal standard.

We observed some oxidized cholesterol derivatives in heated tallow using the present analytical method. Previously, Park and Addis (18) observed the production of 7α- and 7β-hydroxycholesterols, 7-ketocholesterol, and 5α-epoxycholesterol in heated tallow. Bascoul *et al*. (19) also reported *ca.* 25% of cholesterol was destroyed after 60 h of deep frying in tallow and found oxidized cholesterol derivatives such as cholestanetriol, 7α- and 7β-hydroxycholesterols, 7-ketocholesterol, epoxycholesterol, and 7-ketocholesta-3-5 dien in GC analysis. Thus, each oxidized cholesterol derivative observed in our system was consistent with previous results obtained by GC analysis. Moreover, eggnog mix irradiated with fluorescent light in air contained 7α-hydroxycholesterol levels exceeding those of 7β-hydroxycholesterol (20). The tendency of our result was consistent with this report. Van de Bovenkamp *et al*. (21) also showed the qualitative evidence of cholesterol oxidation in study of photooxidation of Dutch foods. Korytowski *et al*. (24) showed the existence of various hydroperoxysterols in photooxidized cholesterol using HPLC-electrochemical detector method. On the other hand, Caboni *et al*. (12) reported the possibility of the analysis of 7α- and 7β-hydroperoxycholesterols using ELSD and cyano-bonded normal-phase column. Therefore, it may be possible to identify oxidized cholesterol derivatives containing cholesterol hydroperoxide by only our analytical method or combination of our system with electrochemical detector because we observed the production of hydroperoxide in thin-layer chromatography analysis of photooxidized cholesterol. On the other hand, oxidized cholesterol derivatives in copper-oxidized LDL were the same for other previous reports using GC analysis (25,26). Thus oxidized cholesterol derivatives observed in previous GC analysis were also identified in our analytical method, although the levels of oxidized cholesterol derivatives were different among all results. This discrepancy may result from the difference of each condition of oxidation.

Finally, we analyzed oxidized cholesterol derivatives by a combination of photodiode-array detection and electrospray MS detection. The 10 available species of oxidized cholesterol derivatives were adequately resolved, and each oxidized cholesterol derivative was also identified by this combined analysis system within 30 min, although our analytical method was inadequate for ionization of cholesterol. Thus, the accurate analysis of oxidized cholesterol derivatives using HPLC/ESI/MS was also performed by our system. However, there is ample room for further improvement in this condition because cholesterol was not ionized in this method. In addition, more detailed studies on the HPLC/ESI/MS analysis of oxidized cholesterol derivatives seem to be required, since the characteristic ion of each oxidized cholesterol derivative standard may contain the contaminants or the unknown ion peaks in our result.

The analytical method using ELSD detects various oxidized cholesterol derivatives with different chemical functional groups and spectrophotometric absorption; however, the sensitivities of detection for 7-ketocholesterol, 3,5-cholestadien-7-one, and 4,6-cholestadien-3-one were higher in UV analysis than in ELSD analysis. Therefore, the simultaneous HPLC analysis by connection of diode-array detector and ELSD may allow the accurate identification of oxidized cholesterol derivatives in various foods and biological specimens.

In conclusion, it is possible to separate 10 species of oxidized cholesterol derivatives and cholesterol within 30 min using a reversed-phase column and methanol/acetonitrile (60:40, vol/vol) as the mobile phase at 1.0 mL/min with UV and ELSD detection. This analysis method showed a liner response for oxidized cholesterol derivatives in the range 0–2000 ng, and minimal limits of detection range from 100 to 500 ng. Moreover, This HPLC analysis with combined UV and ELSD was able to be applied to the determination of oxidized cholesterol derivatives in heated tallow, photooxidized cholesterol, and copper-oxidized LDL.

ACKNOWLEDGMENTS

This work was supported by the Heart and Stroke Foundation of Ontario and Japan Society for the Promotion of Science.

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