# Determination of Sterols, Oxysterols, and Fatty Acids of Phospholipids in Cells and Lipoproteins: A One-Sample Method

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**ABSTRACT:** In addition to fatty acids, especially polyunsaturated species, cholesterol oxidizes and leads to various oxygenated derivatives, named oxysterols. They display a wide range of adverse biological properties. Monitoring oxysterols is important in the evaluation of the potential risks associated with lipid oxidation. In the present study, a quick and reliable method was developed for analysis of oxysterols, sterols, and fatty acid composition of phospholipids in the same biological sample. Total lipid extraction was determined after addition of several internal standards (epicoprostanol for sterols, 19-hydroxy-cholesterol for oxysterol and di-heptadecanoyl-phosphatidylcholine for phospholipid fatty acids). Cold acetone-mediated precipitation was then used to fractionate sterols from phospholipids. The phospholipid-containing precipitate was transmethylated for fatty acid analysis by gas chromatography. The sterol- and oxysterol-containing phase was saponified under mild conditions to avoid artificial oxysterol generation and was analyzed by gas chromatography after derivatization into trimethylsilyl ethers. The overall procedure was found to be specific with good recovery and reproducibility for sterols, oxysterols [mean coefficient of variation in percent (CV), 11.3%] as well as phospholipid fatty acids (CV, 5.6%). This procedure has been used to document *in vitro* free radical treated-human low-density lipoproteins and erythrocytes. Results demonstrated that this method is a useful tool in assessing qualitative and quantitative differences in oxysterols and phospholipid fatty acid patterns attributed to lipid oxidation.
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**KEY WORDS:** Cholesterol oxides, erythrocytes, free radicals, low-density lipoproteins, oxidation, oxysterols, phospholipid fatty acids.

It has become increasingly evident that oxidative damage may contribute to the development of various pathologies (1). In cardiovascular disease, lipid peroxidation is important because oxidative modification of low-density lipoproteins (LDL) is a prerequisite for the enhanced uptake of cholesterol by macrophages (2,3). Unsaturated fatty acids have a propensity to undergo peroxidation. Not only are the fatty acid composition of cell membrane and lipoproteins thus altered but also reactive and potentially damaging end products are produced during the oxidation process. Cholesterol can also be oxidized and produce products called oxysterols (OS). OS can originate from endogenous oxidation such as enzyme-derived cholesterol catabolism and peroxidation of cholesterol-rich lipoproteins (4). In the course of LDL oxidation, cholesterol hydroperoxides are formed (5) and enhance the susceptibility of metal-mediated oxidation of LDL (6). In this process, a great amount of 7-oxidized derivatives were found. OS may also be absorbed from food, as they were identified in some processed foods like egg-containing products and powdered milk (7). In addition to their demonstrated atherogenicity (8), OS also have shown other detrimental properties. OS exhibit cytotoxicity (9), inhibition of hydroxymethyl coenzyme A reductase (10), and increase in cholesterol esters by stimulation of acyl-coenzyme A:cholesterol acyl transferase activity (11). Recent evidence indicated that epoxy cholesterol can function as a direct-acting mutagen and that there might be a relationship between its presence and skin cancer (12).

Consequently, it is of interest to determine the lipid compositions of cells and lipoproteins which can be used as an index for oxidative damage. The objectives of the present paper were to develop a method capable of measuring the OS concentration and the fatty acid pattern of phospholipids in the same sample of cells or lipoproteins.

## EXPERIMENTAL PROCEDURES

*Materials and reagents.* [$^{14}$C]-Cholesterol, 3-oleoyl-[$^{14}$C]-cholesterol ester (CE), 1,2-di[1-$^{14}$C]palmitoyl-L-3-phosphatidylcholine (PC); 1-[1-$^{14}$C]palmitoyl-L-3-phosphatidylcholine (lysoPC, LPC), and glycerol tri-[1-$^{14}$C]oleate (TO) were obtained from Amersham. Cholesterol (5-cholesten-3β-ol), epicoprostanol (5β-cholestan-3α–ol, Epico), β-sitosterol (24β-ethyl-5-cholesten-3β-ol, β-Sito), campesterol (24α-methyl-5-cholesten-3β-ol, Camp), 7α-hydroxycholesterol (5-cholestene-3β,7α-diol, 7α-OH), 19-hydroxycholesterol (5-cholestene-3β,19-diol, 19-OH), 3,5-cholestadiene (Diene), and

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1,2-di-heptadecanoyl-L-3-phosphatidylcholine (C17PC) were obtained from Sigma (St. Quentin-Fallavier, France). 20-Hydroxycholesterol (5-cholestene-3β,20-diol, 20-OH), 22*S*-hydroxycholesterol (5-cholestene-3β,22*S*-diol, 22-OH), 25-hydroxycholesterol (5-cholestene-3β,25-diol, 25-OH), cholesterol epoxides (5,6β-epoxy-5α-cholestan-3β-ol, β-Epox, and 5,6α-epoxy-5α-cholestan-3β-ol, α-Epox), and cholestanetriol (5α-cholestan-3β,5,6β-triol, Triol) were purchased from Steraloids (Wilton, NH). 7-Ketocholesterol (5-cholestene-3β-hydroxy-7-one, 7Keto) was obtained from Aldrich (St. Quentin-Fallavier, France). 7β-Hydroxycholesterol (5-cholestene-3β,7β-diol, 7β-OH) was synthesized by reduction of 7Keto with a 10% solution of $NaBH_4$ in methanol in the presence of $CeCl_2$ (13). When necessary (purity <95%), the OS were repurified by thin-layer chromatography (TLC) on silica gel impregnated plates (Merck, Darmstadt, Germany) with hexane/ethyl acetate (70:30, vol/vol), and purity was checked by GC (see below). Pyridine (dried over molecular sieves) was from Merck, and silylating agents Silon BTZ, *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA), and trimethylchlorosilane (TMCS) were purchased from Supelco (Bellefonte, PA) or Pierce (Rockford, IL). Butylated hydroxytoluene (BHT), disodium EDTA, and sodium citrate were obtained from Sigma. The free radical generator [2,2′-azo-bis(2-amidinopropane hydrochloride), AAPH] was obtained from Lara-Spiral (Dijon, France).

*Biological samples.* After an overnight fast, blood samples were taken from volunteers by venipuncture into evacuated tubes (Becton Dickinson, Grenoble, France) containing disodium EDTA (1.5 mg/mL), and the plasma was separated in a cold centrifuge (Jouan, Nantes, France). LDL ($1.019 < d < 1.055$ g/mL) were isolated by sequential ultracentrifugation with KBr. Pooled LDL were dialyzed against phosphate buffered saline (pH 7.4) containing 100 μM EDTA, sterilized by passage through a Millipore filter (0.22 μm), and stored under argon at 4°C in the dark for no longer than 15 d. Copper-mediated oxidations were performed with 5 μM $CuSO_4$ and 0.2 mg/mL LDL for 6 h at 37°C as previously detailed (14).

Erythrocyte homogenates were prepared by adding washed erythrocyte pellet to water (1:1, vol/vol) containing 100 μM EDTA and frozen at −20°C. Erythrocytes were subjected to the action of free radicals as detailed in Reference 15. Briefly, washed erythrocytes (15% hematocrit) were incubated at 37°C for 90 min in the presence of 100 mM of the free radical generator AAPH. Aliquots of the suspension (0.4 mL) were analyzed for sterols and fatty acid phospholipids as indicated above.

*Lipid extractions and separations.* Before treatment, the following internal standards (solubilized in $CH_2Cl_2/CH_3OH$, 1:1, vol/vol) were added to the samples: 25 μg Epico, 3 μg 19-OH, and 5 μg C17PC. For LDL and platelets, total lipid extractions were carried out according to Folch *et al.* (16) with $CH_2Cl_2$ and 0.005% BHT. For erythrocytes, the method of Rose and Oklander (17) was used. Lipid extracts were evaporated in glass tubes under nitrogen gas. Cold acetone (1 mL, −20°C) was added to the dried residue, vortex-mixed, and sonicated for 20 s and left for at least 30 min on ice. Phospholipids were precipitated and after centrifugation (2000 rpm, 10 min, 4°C), the acetone phase was transferred to a screw-conical glass tube with a teflon-coated cap. The precipitate was washed with cold acetone (0.5 mL) and centrifuged again as before.

*Analysis of sterols and OS.* The acetone phases were evaporated under a stream of nitrogen, and the extract was derivatized to trimethylsilyl (TMS) ether by heating 10 min at 50°C with BTZ in pyridine (30:70, vol/vol). When esterified, sterols were expected in samples, such as in hyperlipemic plasmas. The evaporated acetone phase was saponified with 0.5 mL cold 0.5 M KOH, flushed with argon, and held at room temperature for 2 h. OS were separated by TLC on silica gel with either solvent A [heptane/diethyl ether (1:1, vol/vol)] or solvent B [heptane/ethyl acetate (1:1, vol/vol)].

Gas chromatographic (GC) analyses of sterols and OS were carried out using a DI 200 chromatograph (Delsi, France) equipped with a flame-ionization detector (300°C), a falling needle injector (270°C), and a bonded capillary column (OV1701, 0.1 μm thickness, 0.32 mm i.d., 30 m; Lara-Spiral). The oven temperature was programmed from 260 to 275°C at a rate of 2°/min with helium as carrier gas with a velocity set at 20 cm/s. In some cases, a SE30 capillary column having the same length and characteristics was used. When necessary, samples were analyzed by gas chromatography–mass spectrometry (GC–MS) using a quadrupole spectrometer (Nermag R10-10).

*Analysis of fatty acids.* The acetone-mediated phospholipid precipitate was transmethylated according to Morrisson and Smith (18). Methanol (0.3 mL), toluene (0.3 mL), and $BF_3$/methanol (0.5 mL) were added to the sample. After 90 min at 100°C and cooling on ice, $K_2CO_3$ (2 mL) was added, and the methyl esters were extracted with isooctane (3 mL). After concentration under a nitrogen stream, an aliquot of the volume (0.2–0.6 μL) was injected into the chromatograph. Analyses were performed as described (19) using a DN 200 (Delsi-Nermag, France) equipped with a hydrogen flame detector (260°C) and a cold on-column injector. The chromatograph contained an SP-2340 fused-silica capillary column (30 m × 0.32 mm i.d.; film thickness, 0.2 μm; Supelco). Helium was used as the carrier gas at a linear velocity of 19 cm/s. The oven temperature was programmed at 5°C/min from 90 to 135°C, increased to 220°C at 2°C/min, and held at that temperature for 10 min. Methyl esters were identified by comparison with standard mixtures and quantitated against the heptadecanoate methyl ester recovered from the C17PC.

## RESULTS AND DISCUSSION

*Specificity of the procedure.* To test the specificity and the selectivity of the extraction procedure, red blood cell homogenates obtained by freezing and thawing were supplemented with either [$^{14}$C]-cholesterol, [$^{14}$C]-PC, or [$^{14}$C]-LPC.

**TABLE 1**
**Fractionation of Different Lipids with Acetone Precipitation**

| | Radioactivity recovered | |
|---|---|---|
| | In the acetone phase (%) | In the precipitate (%) |
| Erythrocyte homogenate[a] | | |
| + [$^{14}$C]Chol | 99.6 ± 3.2 | 0.2 ± 0.1 |
| + [$^{14}$C]PC | 0.3 ± 0.1 | 96.1 ± 4.2 |
| + [$^{14}$C]LPC | 0.4 ± 0.2 | 97.2 ± 3.6 |
| + [$^{14}$C]TO | 96.8 ± 3.6 | 0.3 ± 0.1 |
| + [$^{14}$C]CO | 98.5 ± 3.1 | 0.1 ± 0.1 |

[a]Erythrocyte homogenates (0.2 mL) were mixed with the indicated labeled compound (~10,000 dpm, 2 µg) and processed through the entire protocol described in the Experimental Procedures section. The results (mean ± S.D., $n = 5$) were expressed as percentage of the total radioactivity. Abbreviations: Chol, cholesterol; PC, phosphatidylcholine; LPC, lysophosphatidylcholine; TO, triolein; CO cholesteryl oleate.

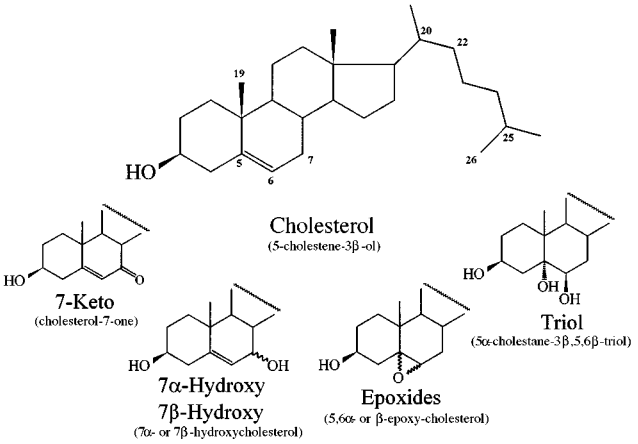


**FIG. 1.** Structure of oxysterols.

Samples were then processed using the entire protocol except the acetone phase was evaporated and dissolved in $CH_2Cl_2/CH_3OH$. The acetone-mediated precipitate was also solubilized in the latter solvent mixture, and aliquot solutions of the two phases were counted for radioactivity. The results (Table 1) indicate that sterols were found, as expected, in the acetone phase, whereas phospholipids were recovered in the precipitate. Sonication and washing of the acetone-mediated precipitate was necessary to obtain a high yield. This resulted in improved recovery for both the sterols and the phospholipids. Table 1 also indicates that triglycerides or cholesterol esters may be present in the acetone phase. It was necessary to saponify these lipids when samples contained large amounts of triglycerides or esterified sterols. Moreover, analyses carried out with diethyl ether resulted in overestimation of the OS concentration, possibly owing to trace amounts of peroxides which may oxidize cholesterol and may generate OS. Structures of OS derivatives are shown in Figure 1.

*Chromatography of sterols and OS.* Preliminary experiments have confirmed that improved resolution and sensitivity were achieved using TMS ethers compared to underivatized sterols (20). The retention times of standard compounds on two columns relative to the internal standard, Epico, are shown in Table 2. All the sterols were provisionally identified by their retention times compared to available standards. Their identities were confirmed using GC–MS by the fragmentation patterns indicated in Table 2. For routine determinations, OV 1701 achieved a better separation. In particular, poor resolutions were obtained between 19-OH and Camp, between α-Epox, 22-OH and 20-OH, and between 7β-OH

**TABLE 2**
**Relative Retention Times and Mass Spectra of Some Sterols and Oxysterols**

| Sterol | Relative retention time[b] | | Electron impact mass spectra: characteristic ions[c] | | | | | |
|---|---|---|---|---|---|---|---|---|
| (TMS)[a] | SE30 | OV1701 | M | M – 15 | M – 90 | Other ions | | |
| Diene | 0.631 | 0.780 | 368 (100) | 353 (46) | — | 247 (52) | 213 (31) | 147 (86) |
| Epico (IS) | 1.000 | 1.000 | 460 (4) | 445 (12) | 370 (100) | 355 (38) | 342 (6) | 215 (62) |
| Chol | 1.150 | 1.164 | 458 (100) | 443 (28) | 368 (78) | 353 (42) | 129 (95) | 329 (82) |
| 7α-OH | 1.188 | 1.080 | 546 (5) | 531 (3) | 456 (100) | 129 (7) | 366 (16) | 229 (12) |
| 19-OH (IS) | 1.321 | 1.268 | — | 531 (<1) | 456 (21) | 366 (67) | 353 (100) | |
| 7β-OH | 1.557 | 1.980 | 546 (8) | 531 (2) | 456 (100) | 129 (6) | 473 (10) | 366 (18) |
| Camp | 1.352 | 1.414 | 472 (54) | — | 382 (48) | 367 (12) | 343 (38) | |
| 22-OH | 1.476 | 1.476 | — | — | 546 (4) | 382 (2) | 343 (4) | |
| 20-OH | 1.495 | 1.596 | 462 (8) | 447 (2) | 372 (6) | 282 (2) | 267 (3) | |
| β-Epox | — | 1.512 | 474 (100) | 459 (21) | 384 (61) | 446 (8) | 369 (11) | 366 (28) |
| α-Epox | 1.423 | 1.628 | 474 (100) | 459 (18) | 384 (56) | 446 (6) | 369 (10) | 366 (34) |
| β-Sito | 1.568 | 1.667 | 486 (52) | 471 (11) | 396 (100) | 381 (31) | 357 (63) | 329 (4) |
| 7Keto | 1.692 | 1.702 | 472 (100) | 457 (28) | 382 (11) | 367 (27) | 341 (2) | |
| 25-OH | 1.755 | 1.934 | 546 (6) | 531 (2) | 456 (6) | 441 (4) | 271 (14) | 131 (100) |
| Triol | 1.833 | 1.969 | 564 (3) | — | 456 (4) | 271 (7) | 131 (100) | 321 (23) |

[a]Abbreviations: TMS, trimethylsilyl ethers; Diene, 3,5-cholestadiene; Epico, epicoprostanol; 7α-OH, 7α-hydroxycholesterol; 19-OH, 19-hydroxycholesterol; 7β-OH, 7β-hydroxycholesterol; Camp, campesterol; 22-OH, 22*S*-hydroxycholesterol; 20-OH, 20-hydroxycholesterol; β-Epox, 5,6β-epoxy-5α-cholestan-3β-ol; α-Epox, 5,6α-epoxy-5α-cholestan-3β-ol; β-Sito, β-sitosterol; 7Keto, 7-ketocholesterol; 25-OH, 25-hydroxycholesterol; Triol, cholestanetriol; IS, internal standard. For other abbreviation see Table 1.

[b]The absolute retention time of Epico was typically 15 min under the conditions used (OV1701, see the Experimental Procedures section). It was 19 min for 19-OH. Each relative retention time varied less than 2%.

[c]Percentage of base peak in parentheses.

and β-Sito. In biological samples, several internal standards were used. Epico was used to quantitate cholesterol and phytosterols, which were the major sterols. For minor sterols, such as OS, 19-OH was used because it was usually absent from biological samples. It is now considered that the high amount of 19-OH reported by Higley *et al.* (21) in meat products was unusual and questionable (7). Resolution was complete even at cholesterol concentrations 50-fold higher than each of OS, a realistic situation in biological or food product samples. Each sterol gave one peak under these experimental conditions. Such was not the case for other silylating agents, which are less powerful, or when the derivatizing temperature exceeded 50°C. In those cases, several peaks might be obtained, especially for Triol, 7Keto or 20-OH.

*Recovery, linearity and reproducibility.* Recovery of the overall extraction technique was assessed by adding various amounts of OS to erythrocyte homogenates (containing negligible amounts of OS) with a fixed amount of the internal standards, Epico and 19-OH. Samples were processed through the entire procedure and finally analyzed by GC. Excellent linear responses were recorded for each sterol in varying weight ratios from 0 to 2. Results of the response factors and of the recoveries are summarized in Table 3. This table shows that the usually low recovery reported for Triol was not observed, possibly because TLC was not used in our isolation procedure. These data indicate that the acetone phase contained not only the sterols, as stated above, but also OS. These results may be explained by the use of 19-OH as internal standard, instead of commonly used α-cholestane. Epico has only one silylable OH as does cholesterol, Camp and β-Sito whereas 19-OH has two OH groups as do most OS. Because of chemical structural similarities with the compounds of interest, the behavior of Epico and 19-OH are much more representative of the losses that might occur during the isolation procedure.

When esterified OS are expected, samples must be saponified. Mild conditions have to be used to minimize artificial generation of OS. Hydrolysis performed at high temperature with high concentrations of KOH (>0.8 M) resulted in a loss of several OS, especially 7Keto (20,22,23). In taking this observation into account, our proposed procedure used mild conditions, although more than 90% hydrolysis was reached based on the saponification of cholesterol esters. For estimation of artificial oxidation of cholesterol, purified [$^{14}$C]-cholesterol (5 μg) was added to samples which were then subjected to the entire protocol except that the OS-containing acetone phase was spotted to silica gel TLC as detailed in the Experimental Procedures section (solvent A). The results indicate that no significant oxidation occurred during the extraction stage, since less than 0.3% of the applied radioactivity was recovered in the OS area ($R_f$ 0.0–0.17). The precision of the method was determined on pooled samples of homogenates of erythrocytes spiked with various OS. We found a good reproducibility since a mean coefficient of variation (CV) of 11.3% was obtained (range 3.1–23.2%) depending of the OS. The detection limit was found to be in the 0.5–2 ng range, depending on the OS, on the basis of the recorded signal-to-noise ratio.

*Specificity, recovery and reproducibility of fatty acids in phospholipids.* To determine whether the phospholipid composition of the acetone precipitate was representative of the analyzed erythrocytes, both the total lipid extract and the acetone precipitate were subjected to phospholipid analysis. This was carried out by means of high-performance liquid chromatography (HPLC) using a light-scattering detector (24). Results showed no significant difference between the two procedures.

Qualitative and quantitative comparisons of the fatty acid composition of the total phospholipids were also performed. By means of the internal standard di-heptadecanoate phosphatidylcholine (C17PC), we found that losses of fatty acids were much more important (by 19.6%, $P < 0.005$) using the classical transmethylation of the TLC-isolated phospholipid fraction. This could be explained by greater losses during the classical TLC procedure and especially by a low elution of phospholipids from silica gel. Furthermore, the fatty acid composition analyzed after the TLC procedure showed a significant loss of polyunsaturated fatty acids (PUFA), especially the long-chain n-3 fatty acids, compared to our proposed method (Table 4). This could be explained by the length of the TLC method, which could expose PUFA to oxidation. Conversely, by means of our procedure, the phospholipid fraction is much more quickly worked up, resulting in a diminished chance to oxidize.

*LDL oxidation*. The lipid composition of LDL was analyzed before and after copper-mediated oxidation *in vitro*. After processing using our procedure, we confirmed that both the sterol fractions and the fatty acid composition of the phospholipids of the LDL were modified after oxidation. Results (Table 5) indicated that the total cholesterol content diminished by 30%, and that various OS appeared. The 7-deriva-

**TABLE 3**
**Relative Response Factors and Recoveries for TMS Ethers of Sterols and Oxysterols[a]**

| Sterol | Response factors | Recovery |
|---|---|---|
| Epico (IS) | 1.00 | 100.0 |
| Chol | 0.96 ± 0.01 | 101.4 ± 2.9 |
| Camp | 0.96 ± 0.01 | 102.3 ± 3.1 |
| β-Sito | 0.98 ± 0.01 | 101.8 ± 3.4 |
| 19-OH (IS) | 1.00 | 100.0 |
| Diene | 1.12 ± 0.04 | 104.1 ± 3.4 |
| 7α-OH | 1.04 ± 0.03 | 100.8 ± 6.1 |
| 7β-OH | 0.98 ± 0.05 | 101.2 ± 4.3 |
| 22-OH | 1.05 ± 0.04 | 96.1 ± 5.8 |
| 20-OH | 1.09 ± 0.05 | 102.3 ± 2.1 |
| α-Epox | 1.03 ± 0.07 | 101.1 ± 3.7 |
| β-Epox | 1.02 ± 0.06 | 99.6 ± 4.2 |
| 7Keto | 1.16 ± 0.04 | 98.4 ± 3.8 |
| 25-OH | 1.12 ± 0.03 | 97.6 ± 4.1 |
| Triol | 0.94 ± 0.06 | 94.7 ± 5.3 |

[a]See Tables 1 and 2 for abbreviations.

**TABLE 4**
**Analysis of Fatty Acids of Phospholipids: Comparison of Two Methods**

| | Reference TLC–GC procedure | | Proposed procedure | |
|---|---|---|---|---|
| Fatty acid | Mean ± SD (nmol)[a] | CV (%) | Mean ± SD (nmol) | CV (%) |
| 16:0 DMA | 2.06 ± 0.1 | (4.9) | 2.28 ± 0.07 | (3.1) |
| 16:0 | 22.31 ± 0.51 | (2.3) | 17.62 ± 0.33[b] | (1.9) |
| 18:0 DMA | 4.23 ± 0.18 | 4.3 | 5.06 ± 0.36[c] | (7.1) |
| 18:0 | 16.39 ± 1.49 | 9.1 | 17.39 ± 0.76 | (4.4) |
| 18:1 | 16.86 ± 0.51 | 3.0 | 14.87 ± 0.39[c] | (2.2) |
| 18:2n-6 | 13.46 ± 0.48 | 3.6 | 11.31 ± 0.31[b] | (2.4) |
| 18:3n-6 | 0.09 ± 0.08 | 88.8 | 0.14 ± 0.04 | (2.7) |
| 18:3n-3 | 0.17 ± 0.06 | 35.3 | 0.11 ± 0.02 | (28.5) |
| 20:3n-6 | 1.43 ± 0.25 | 17.5 | 1.51 ± 0.20 | (18.2) |
| 20:4n-6 | 11.51 ± 0.24 | 2.1 | 14.77 ± 0.49[b] | (13.2) |
| 20:5n-3 | 0.49 ± 0.09 | 18.4 | 0.64 ± 0.07 | (3.3) |
| 22:4n-6 | 2.11 ± 0.17 | 8.1 | 2.56 ± 0.07 | (2.7) |
| 22:5n-3 | 2.52 ± 0.13 | 5.2 | 4.53 ± 0.44[b] | (9.7) |
| 22:6n-3 | 4.76 ± 0.21 | 4.4 | 7.05 ± 0.14[b] | (2.0) |
| Total (nmol) | 1854 ± 228 | 12.3 | 2306 ± 129[b] | (5.6) |

[a]Values (means ± SD, $n = 5$) are expressed as nmol% for each fatty acid and as nmoles/0.2 mL erythrocyte homogenate for total fatty acids. Abbreviations: CV, coefficient of variation as a percentage; DMA, dimethylacetal; TLC–GC, thin-layer chromatography–gas chromatography.
[b]Statistical analysis (unpaired Student's *t*-test): $P < 0.01$.
[c]Statistical analysis (unpaired Student's *t*-test): $P < 0.005$.

**TABLE 5**
**Analysis of Sterols, Oxysterols, and Phospholipid Fatty Acids (PL–FA) of Native and Copper-Oxidized Low-Density Lipoproteins (LDL)**

| | Native LDL[a] | Oxidized LDL |
|---|---|---|
| Sterols (μmol/mg protein) | | |
| Chol | 3850 ± 127 | 2710 ± 188[b] |
| Camp | 552 ± 38 | 487 ± 51 |
| β-Sito | 114 ± 13 | 98 ± 9 |
| Oxysterols (nmol/mg protein) | | |
| 7α-OH | 9.9 ± 0.3 | 13.8 ± 0.3[b] |
| 7β-OH | nd | 56.4 ± 2.2[b] |
| 7Keto | 13.7 ± 0.2 | 43.2 ± 1.8[b] |
| α-Epox | 4.5 ± 0.1 | 62.6 ± 3.6[b] |
| Triol | nd | 41.3 ± 2.3[b] |
| 25-OH | nd | 12.4 ± 2.1[b] |
| PL–FA (nmol/mg protein) | | |
| 16:0 | 327.8 ± 11.4 | 316.6 ± 9.6 |
| 16:1n-7 | 58.2 ± 2.8 | 36.8 ± 2.8[b] |
| 18:0 | 101.5 ± 4.3 | 94.2 ± 4.6 |
| 18:1n-9 | 351.1 ± 10.8 | 321.4 ± 8.6[c] |
| 18:2n-6 | 604.0 ± 14.6 | 458.9 ± 7.9[b] |
| 20:3n-6 | 31.6 ± 2.6 | 2.6 ± 0.4[b] |
| 20:4n-6 | 109.8 ± 4.1 | 17.2 ± 0.9[b] |
| 20:5n-3 | 16.6 ± 1.8 | nd |
| 22:6n-3 | 24.9 ± 2.3 | 1.3 ± 0.2[b] |
| Total | 1664 ± 56 | 1248 ± 64[b] |

[a]Human LDL were incubated with 5 μM $CuSO_4$ at 37°C for 6 h as detailed in the Experimental Procedures section. Sterols, oxysterols and PL-FA were analyzed using the presently described procedure. Results (mean ± SD, $n = 3$) were expressed as indicated. For abbreviations see Tables 1 and 2.
[b]Statistical analysis (unpaired Student's *t*-test): $P < 0.001$.
[c]Statistical analysis (unpaired Student's *t*-test): $P < 0.02$.

**TABLE 6**
**Analysis of Sterols, Oxysterols, and PL–FA of Erythrocytes Before and After *In Vitro* Treatment with AAPH-Free Radicals**

| | Before | After |
|---|---|---|
| Sterols (μmol/$10^9$) | | |
| Chol | 92.3 ± 11.3 | 81.2 ± 12.8 |
| Camp | 2.2 ± 0.2 | 1.8 ± 0.3 |
| β-Sito | 1.1 ± 0.1 | 0.8 ± 0.1 |
| Oxysterol (nmol/$10^9$) | | |
| 7α-OH | 7.9 ± 3.5 | 16.6 ± 4.5 |
| 7β-OH | 31.3 ± 8.0 | 63.3 ± 11.3[b] |
| 7Keto | 293.5 ± 40.6 | 1958.7 ± 55.8[c] |
| α-Epox | 60.5 ± 7.9 | 278.6 ± 13.0[c] |
| β-Epox | 70.7 ± 10.1 | 355.2 18.2[c] |
| Triol | 8.9 ± 3.0 | 102.8 ± 10.6[c] |
| 22-OH | 38.5 ± 11.0 | 136.2 ± 18.9[c] |
| 25-OH | 27.9 ± 3.5 | 103.2 ± 8.2[c] |
| Diene | 138.6 ± 17.3 | 206.8 ± 31.0[b] |
| PL–FA (nmol%) | | |
| 16:0 | 18.32 ± 0.32 | 26.84 ± 0.68[c] |
| 18:0 | 18.56 ± 0.64 | 24.64 ± 0.84[c] |
| 18:1n-9 | 15.68 ± 0.39 | 14.81 ± 0.61 |
| 18:2n-6 | 11.87 ± 0.41 | 7.12 ± 0.67[c] |
| 20:4n-6 | 15.84 ± 0.39 | 4.91 ± 0.51[c] |
| 22:4n-6 | 2.81 ± 0.10 | 0.82 ± 0.21[c] |
| 22:5n-3 | 3.74 ± 0.38 | 0.21 ± 0.17[c] |
| 22:6n-3 | 6.82 ± 0.12 | 0.61 ± 0.28[c] |

[a]Human erythrocytes were incubated with 100 mM AAPH [2,2′-azo-bis(2-amidinopropane hydrochloride)] at 37°C as detailed in Experimental Procedures. After 90 min incubation, sterols, oxysterols, and PL–FA were analyzed using the presently described procedure. Results (mean ± SD, $n = 3$) were expressed as indicated. For other abbreviations see Tables 1, 2, and 5.
[b]Statistical analysis (unpaired Student's *t*-test): $P < 0.05$.
[c]Statistical analysis (unpaired Student's *t*-test): $P < 0.001$.

tives of OS were most abundant but epoxide, Triol, and 25-OH could be identified. Our results confirmed the data reported in other similar studies (9,25) although no attempt was made to characterize the free and the esterified forms (26). Concerning the fatty acid composition, we found that a loss of phospholipids occurred during the oxidation process (–25%) and was attributed to loss of the long-chain PUFA.

*Free radical-treated erythrocytes.* Erythrocytes were incubated with the free radical generator, AAPH. This technique has been used to determine the susceptibility of animals or humans to free radical attack (15,27,28). Table 6 shows that 90 min after the oxidative treatment, the lipid composition of erythrocytes was markedly modified. The cholesterol content decreased, as well as Camp and β-Sito. Trace amounts of OS were identified before oxidation, whereas various OS were quantitated after *in vitro* free radical treatment. The fatty acid pattern of the erythrocytes, primarily PUFA, also changed before and after oxidation.

Existing methods for determining the effect of oxidation processes on lipid parameters are often time-consuming and have variable recovery coefficients. Previous studies have demonstrated that artificial oxidation of cholesterol or fatty acids may occur during processing (29). This could be due to the use of tedious solvent extraction, column or TLC fractionation which exposed sterols or fatty acids to air/light oxidation. Our procedure is able to determine both the sterol and OS composition and the fatty acid pattern of phospholipids on the same biological sample. Although antioxidants were added (EDTA, BHT), this method reduces storage time which may generate artificial OS (30,31) and denature fatty acids.

In summary, the procedure described in the present paper is simple, efficient, and accurate. It allows one to analyze sterols, OS, and the fatty acid pattern of phospholipids in a qualitative and quantitative manner and hence to document the mechanisms of the oxidation processes in atherogenesis.

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## REFERENCES

1. Halliwell, B., and C.E. Cross, Oxygen-Derived Species: Their Relation to Human Disease and Environmental Stress, *Environ. Health Perspect. 102 Suppl. 10:*5–12 (1994).
2. Esterbauer, H., J. Gebicki, H. Puhl, and G. Jürgens, The Role of Lipid Peroxidation and Antioxidants in Oxidative Modification of LDL, *Free Radical Biol. Med. 13:*341–390 (1992).
3. Witztum, J.L., and D. Steinberg, Role of Oxidized Low Density Lipoprotein in Atherogenesis, *J. Clin. Invest. 88:*1785–1792 (1991).
4. Smith, L.L., and B.H. Johnson, Biological Activities of Oxysterols, *Free Radical Biol. Med. 7:*285–332 (1989).
5. Malavasi, B., M.F. Rasetti, P. Roma, R. Fogliatto, P. Allevi, A.L. Catapano, and G. Galli, Evidence for the Presence of 7-Hydroperoxycholest-5-en-3β-ol in Oxidized Human LDL, *Chem. Phys. Lipids 62:*209–214 (1992).
6. Blache, D., C. Rodriguez, and J. Davignon, Pro-Oxidant Effects of 7-Hydroperoxycholest-5-en-3β-ol on the Copper-Initiated Oxidation of Low Density Lipoprotein, *FEBS Lett. 357:*135–139 (1995).
7. Paniangvait, P., A.J. King, A.D. Jones, and B.G. German, Cholesterol Oxides in Foods of Animal Origin, *J. Food Sci. 60:*1159–1174 (1995).
8. Peng, S.-K., B. Hu, and R.J. Morin, Angiotoxicity and Atherogenicity of Cholesterol Oxides, *J. Clin. Lab. Anal. 5:*144–152 (1991).
9. Sevanian, A., H.N. Hodis, J. Hwang, L.L. McLeod, and H. Peterson, Characterization of Endothelial Cell Injury by Cholesterol Oxidation Products Found in Oxidized LDL, *J. Lipid Res. 36:*1971–1986 (1995).
10. Kandutsch, A.A., H.W. Chen, and H. Heiniger, Biological Activity of Some Oxygenated Sterols, *Science 201:*498–501 (1978).
11. Zhang, H., H.J.K. Basra, and U.P. Steinbrecher, Effects of Oxidatively Modified LDL on Cholesterol Esterification in Cultured Macrophages, *J. Lipid Res. 31:*1361–1369 (1990).
12. Morin, R.J., B. Hu, S.-K. Peng, and A. Sevanian, Cholesterol Oxides and Carcinogenesis, *J. Clin. Lab. Anal. 5:*219–225 (1991).
13. Kumar, V., A. Amann, G. Ourisson, and B. Luu, Stereospecific Synthesis of 7β-Hydroxycholesterol and 7α-Hydroxycholesterol, *Synth. Comm. 17:*1229–1286 (1987).
14. Blache, D., C. Rodriguez, and J. Davignon, Enhanced Susceptibility of Cholesteryl Sulfate-Enriched Low Density Lipoproteins to Copper-Mediated Oxidation, *FEBS Lett. 362:*197–200 (1995).
15. Durand, P., and D. Blache, Enhanced Platelet Thromboxane Synthesis and Reduced Macrophage-Dependent Fibrinolytic Activity Related to Oxidative Stress in Oral Contraceptive-Treated Female Rats, *Atherosclerosis 121:*205–216 (1996).
16. Folch, J., M. Lees, and G.H. Sloane-Stanley, A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissues, *J. Biol. Chem. 226:*497–509 (1957).
17. Rose, H.G., and M. Oklander, Improved Procedure for the Extraction of Lipids from Human Erythrocytes, *J. Lipid Res. 6:*428–431 (1965).
18. Morrisson, W.R., and L.M. Smith, Preparation of Fatty Acid Methyl Esters and Dimethyl Acetates from Lipids with Boronfluoride Methanol, *Ibid. 5:*600–608 (1964).
19. Polette, A., P. Durand, B. Floccard, and D. Blache, A Method for Specific Analysis of Free Fatty Acids in Biological Samples by Capillary Gas Chromatography, *Anal. Biochem. 206:*241–245 (1992).
20. Won Park, S., and P.B. Addis, Capillary Column Gas–Liquid Chromatographic Resolution of Oxidized Cholesterol Derivatives, *Ibid. 149:*275–283 (1985).
21. Higley, N.A., S.L. Taylor, A.M. Herian, and K. Lee, Cholesterol Oxides in Processed Meats, *Meat Sci. 16:*175–188 (1986).
22. Van de Bovenkamp, P., T.G. Kosmeijer-Schuil, and M.B. Katan, Quantification of Oxysterols in Dutch Food: Egg Products and Mixed Diets, *Lipids 23:*1079–1085 (1988).
23. Dzeletovic, S., O. Breuer, E. Lund, and U. Diczfalusy, Determination of Cholesterol Oxidation Products in Human Plasma by Isotope Dilution–Mass Spectrometry, *Anal. Biochem. 225:*73–80 (1995).
24. Juanéda, P., G. Rocquelin, and P.O. Astorg, Separation and Quantification of Heart and Liver Phospholipid Classes by High–Performance Liquid Chromatography Using a New Light-Scattering Detector, *Lipids 25:*756–759 (1990).
25. Dzeletovic, S., A. Babiker, E. Lund, and U. Diczfalusy, Time Course of Oxysterol Formation During *In Vitro* Oxidation of

Low Density Lipoprotein, *Chem. Phys. Lipids 78:*119–128 (1995).
26. Brown, A.J., R.T. Dean, and W. Jessup, Free and Esterified Oxysterol: Formation During Copper-Oxidation of Low Density Lipoprotein and Uptake by Macrophages, *J. Lipid Res. 37:*320–335 (1996).
27. Durand, P., M. Prost, and D. Blache, Pro-thrombotic Effects of a Folic Acid Deficient Diet in Rat Platelets and Macrophages Related to Elevated Homocysteine and Decreased n-3 Polyunsaturated Fatty Acids, *Atherosclerosis 121:*231–243 (1996).
28. Blache, D., and M. Prost, Free Radical Attack: Biological Test for Human Resistance Capability, in *Proceedings of the IX College Park Colloquium on Chemical Evolution: A Lunar-Based Chemical Analysis Laboratory (LBCAL)*, edited by C. Ponnamperuma, and C.W. Gehrke, NASA, Washington, D.C., 1992, pp. 82–98.
29. Smith, L.L., Cholesterol Autoxidation, *Chem. Phys. Lipids 44:*87–125 (1987).
30. Pie, J.E., K. Spahis, and C. Seillan, Evaluation of Oxidative Degradation of Cholesterol in Food and Food Ingredients: Identification and Quantification of Cholesterol Oxides, *J. Agric. Food Chem. 38:*973–979 (1990).
31. Rose-Salin, C., A.C. Huggett, J.O. Bosset, R. Tabacchi, and L.B. Fay, Quantification of Cholesterol Oxidation Products in Milk Powders Using [$^2H_7$]Cholesterol to Monitor Autoxidation Artifacts, *Ibid. 43:*935–941 (1995).

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