Ion-trap tandem mass spectrometric analysis of squalene monohydroperoxide isomers in sunlight-exposed human skin

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Abstract   We previously discovered that squalene mono-hydroperoxide (SQ-OOH) was produced on human fore-head skin and suggested that skin squalene (SQ) may be the principal target lipid for oxidative stress (e.g., sunlight exposure). Because of its six double bonds, SQ peroxi-dation can yield various positional hydroperoxide isomers. However, the structural characterization of skin SQ-OOH isomers has never been reported. Here, we prepared pure SQ-OOH isomers and developed an analytical method for SQ-OOH isomers using a quadrupole/linear ion-trap mass spectrometer (QTRAP) MS/MS system. Collision-induced dis-sociation produced specific fragment ions for each SQ-OOH isomer, which permitted discrimination between SQ-OOH isomers by multiple reaction monitoring (MRM). When lipid extract from human forehead skin was subjected to LC-MS/MS with MRM, individual SQ-OOH isomers could be sepa-rated and detected with a sensitivity of 0.05 ng/injection. The total concentration of SQ-OOH isomers in forehead skin was ~956 μg/g skin lipids, but it increased up to 2,760 μg/g skin lipids after 3 h of sunlight exposure. The LC-MS/MS method was useful for investigating the peroxidation mecha-nisms of SQ as well as SQ-OOH-mediated skin disorders.—Nakagawa, K., D. Ibusuki, Y. Suzuki, S. Yamashita, O. Higuchi, S. Oikawa, and T. Miyazawa. **Ion-trap tandem mass spectro-metric analysis of squalene monohydroperoxide isomers in sunlight-exposed human skin.** *J. Lipid Res.* 2007. **48:** 2779–2787.

Supplementary key words   lipid peroxidation • QTRAP mass spec-trometer • skin pathogenicity

Human skin, covering the entire outside of the body, is the largest organ and is exposed constantly to sunlight stress, including ultraviolet (UV) light irradiation (1–3). Because the skin is rich in lipids, consisting mainly of squalene (SQ), triacylglycerol, wax esters, and sterols (4), skin surface lipids are thought to be vulnerable to oxida-tive stress from sunlight.

Previously, we established a liquid chromatography-chemiluminescence detection (LC-CL) method for the sen-sitive and selective determination of lipid hydroperoxide molecules (5, 6) and discovered that SQ is the principal target lipid for peroxidation on the human skin surface (7, 8). SQ is a structurally unique triterpene compound (**Fig. 1**) that is an intermediate in the cholesterol biosyn-thesis pathway (9). The presence of six double bonds al-lows SQ to undergo photooxidation, yielding squalene monohydroperoxide (SQ-OOH) as the primary oxidation product (10). We and other researchers reported that using LC-CL, or the rather insensitive LC-UV detection method at 210 nm, SQ-OOH levels increased markedly in skin sur-face lipids from human forehead after exposure to sunlight (11) or UV A and B (12–14). These studies (7, 8, 10–14) suggested that SQ-OOH accumulation could be involved in inflammatory skin disorders such as skin cancer, cuta-neous autoimmune disease, and skin aging.

Despite the potential significance of SQ-OOH in skin pathology (15), detailed structural characterization of hu-man skin SQ-OOH has never been carried out. Although Mudiyanselage et al. (16) recently reported the analysis of SQ-OOH in UV-exposed human sebum using LC, MS, and NMR, the position of the hydroperoxide group in the SQ-OOH molecule was not referred to because of diffi-culties in preparing reliable SQ-OOH standards and the limitations of their analytical method. Therefore, an alter-native method for the complete structural characterization of SQ-OOH in vivo is required to understand the mech-anisms of SQ peroxidation as well as SQ-OOH-mediated skin disorders.
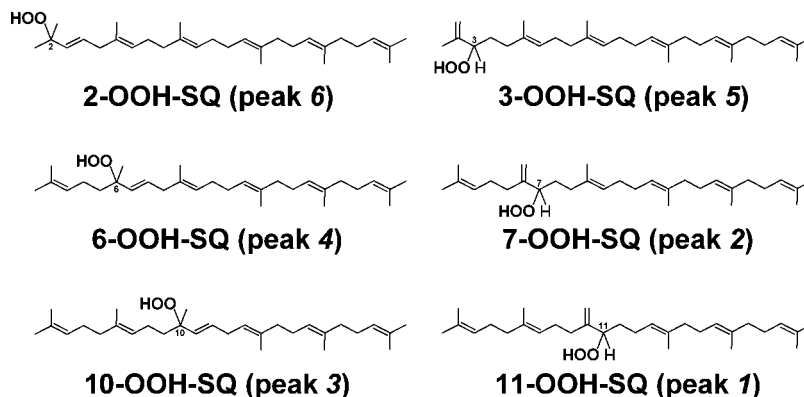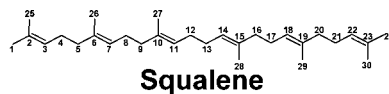
Fig. 1. Chemical structures of squalene (SQ) and six squalene monohydroperoxide (SQ-OOH) isomers (2-OOH-SQ, 3-OOH-SQ, 6-OOH-SQ, 7-OOH-SQ, 10-OOH-SQ, and 11-OOH-SQ).

The recently developed hybrid quadrupole/linear ion trap (QqLIT) spectrometer, QTRAP, offers specific benefits for LC-MS/MS for biomolecular analysis (17, 18). With the advent of QTRAP, both triple quadrupole and ion-trap scans can be performed together as a single stage. The product ion scan, multiple reaction monitoring (MRM), and neutral loss scan provide useful structural information about the analyte, even in the presence of background contaminants from complex biological matrices. The structural characterization of a variety of lipids, including sphingolipids, phospholipids, and glycated lipids, using QTRAP has been reported (19, 20).

In the present study, we prepared six SQ-OOH isomer standards, with each isomer differing in the position of the hydroperoxide group (Fig. 1). Using these standards, we developed a QTRAP LC-MS/MS method for determining SQ-OOH isomers in human skin surface lipids, and we discuss the possible mechanisms of SQ peroxidation in vivo as well as the pathogenicity of skin SQ-OOH.

MATERIALS AND METHODS

Materials

SQ and cytochrome c were purchased from Sigma (Tokyo, Japan). Acetone, 1-butanol, and luminol were obtained from Wako (Osaka, Japan). All other reagents were of the highest grade available.

Preparation of standard SQ-OOH isomers

The six standard SQ-OOH isomers (Fig. 1) were synthesized from SQ as follows. SQ [100 mg (240 μmol)] was weighed, dissolved in 50 ml of ethanol (containing 0.01 mg/ml rose bengal as a sensitizer) in a test tube, and exposed to oxygen gas for 10 s. The tube was capped and then photooxidized with a 100 W tungsten lamp (Matsushita Electric Industrial Co., Osaka, Japan) at 4°C for 6 h (21). The lamp was held 10 cm vertically above the test tube. After photooxidation, a portion (5 ml) of the resultant solution was loaded onto an ethanol-equilibrated SepPak® Plus QMA cartridge (Waters, Milford, MA). The cartridge was eluted with an additional 5 ml of ethanol, and the eluate containing SQ-OOH moieties was collected. During the SepPak procedure, the rose bengal was retained on the cartridge. The SepPak procedure was repeated 10 times. Finally, the collected eluates were combined, evaporated, and redissolved in 3 ml of ethanol.

A small portion (1 μl) of the ethanolic sample solution was subjected to LC-CL with on-line MS (LC-CL-MS) (22) to monitor the yield of SQ-OOH. For LC-CL-MS, an ODS column (CAPCELLPAK C18, 4.6 × 250 mm; Shiseido, Tokyo, Japan) was used with methanol at a flow rate of 1 ml/min. At the post column, the eluate was split. One portion (flow rate, 0.95 ml/min) was mixed with a hydroperoxide-specific CL reagent (a mixture of cytochrome c and luminol in 50 mM borate buffer, pH 9.3) (5–8, 22–24) and was sent to a JASCO 825-CL detector (Japan Spectroscopic Co., Tokyo, Japan). The flow rate of the CL reagent was 0.9 ml/min. The other portion (flow rate, 0.05 ml/min) was sent to a Mariner ESI time-of-flight mass spectrometer (Applied Biosystems, Foster City, CA). Positive ion spectra were taken in the m/z range 300–1,000.

SQ-OOH was then isolated from the ethanolic sample solution (3 ml) using the semipreparative JASCO LC-UV (210 nm) system with an ODS column (CAPCELLPAK C18, 20 × 250 mm; Shiseido). The ODS column was eluted with methanol at a flow rate of 10 ml/min. The isolated SQ-OOH (a mixture of the six SQ-OOH isomers) was evaporated to dryness and redissolved in 1 ml of cyclohexane. The cyclohexane solution was further subjected to JASCO LC-UV (210 nm) with a silica column (SG 120A, 4.6 × 250 mm; Shiseido) to isolate each individual SQ-OOH isomer. The silica column was eluted with a mixture of cyclohexane-diethylether (100:2, v/v) at a flow rate of 1 ml/min. The structure and purity of the obtained SQ-OOH isomers were evaluated by LC-CL-MS as described above. In addition, each SQ-OOH isomer (2 mg/1 ml CDCl₃) was subjected to NMR, and ¹H and ¹³C NMR spectra were recorded on a Varian (Palo Alto, CA) Unity 600 spectrometer (600 MHz for ¹H NMR and 150 MHz for ¹³C NMR) at 20°C. Chemical shifts were given in ppm relative to tetramethylsilane as an internal standard. Two-dimensional NMR, ¹H-¹H correlation spectroscopy, heteronuclear multiple quantum

correlation, heteronuclear multiple bond correlation, and distortionless enhancement by polarization transfer were performed.

Human skin samples and lipid extraction

Eight healthy male human volunteers (mean age, 20.4 ± 1.4 years) participated in this study. All subjects gave written informed consent to the experimental protocol, which was approved by the local research ethics committee. The study was conducted on September 20, 2006 (a sunny day). Before and after 3 h of sunlight exposure (1,000–2,000 $\mu W/cm^2$), an acetone-wet cotton pad (10 × 10 cm) was placed on the forehead, wiped onto the skin surface five times, and then removed gently to collect skin surface lipids (25). The cotton pads were transferred to tubes containing 30 ml of acetone for lipid extraction (26). After centrifugation at 1,000 g for 5 min at 4°C, acetone layers (lipid fraction) were collected. This lipid extraction with acetone was carried out twice. The combined acetone layers (i.e., total skin surface lipids) were evaporated, and the dried extract was redissolved in 200 μl of 1-butanol. A portion (10 μl) of the extract was subjected to LC-MS/MS (as described below) for the structural determination of individual skin surface SQ-OOH isomers.

LC-MS/MS instrument

The QTRAP LC-MS/MS system consisted of a Shimazu liquid chromatograph (Kyoto, Japan), including a vacuum degasser, quaternary pump, and autosampler, and an Applied Biosystems 4000 QTRAP tandem mass spectrometer equipped with a turbo ion spray source. This instrument used a triple quadrupole ion path in which the final quadrupole was used as a QqLIT mass spectrometer.

Determination of skin surface SQ-OOH isomers by LC-MS/MS

A stock solution of SQ-OOH [4 mg (9 μmol)/ml] was prepared from each standard SQ-OOH isomer dissolved in 1-butanol and stored at −80°C until analysis. We have verified that the stock solution remains stable for up to 3 months under such storage conditions. Before determination of skin surface SQ-OOH, we prepared standard SQ-OOH solutions at concentrations of 0.01, 0.1, 1.0, 2.0, and 4.0 μg/ml (a range expected to encompass concentrations encountered in vivo). Aliquots (10 μl) of these solutions (containing 0.1–40 ng of SQ-OOH) were subjected to LC-MS/MS, and calibration curves were made.

Skin lipid extracts or SQ-OOH standards (10 μl each) were separated on a silica column (Inertsil, 2.1 × 150 mm; GL Science, Tokyo, Japan), eluted with a mixture of cyclohexane-diethylether (100:2, v/v) at a flow rate of 0.5 ml/min, and maintained at 40°C. At the post column, SQ-OOH isomers were individually detected by LC-MS/MS with MRM for transition of the parent ion to the product ion. The concentration of each skin surface SQ-OOH isomer was then calculated according to the calibration curves. For QTRAP MS/MS, atmospheric pressure chemical ionization was used as the ion source, the collision energy for transition was set at 15 eV, the dwell time for transition was 100 ms, the turbo gas temperature was kept at 200°C, and the spray voltage was 5,000 V. Nitrogen values for the turbo, nebulizer, and curtain gases were set at 70, 50, and 20 pounds per square inch, respectively. Positive ion spectra were taken in the m/z range 50–450.

Other assays

The composition of skin surface lipids was analyzed by TLC on Silica Gel G plates (20 × 20 cm glass plates, 0.25 mm thick layer; Merck, Darmstadt, Germany) (27). Aliquots (5 μl) of the skin lipid extracts were applied to the TLC plates, which were then developed with hexane (to 17 cm), benzene (to 17 cm), and finally hexane-ether-acetic acid (70:30:1, v/v/v; to 9 cm). Lipid zones were visualized using methanolic sulfuric acid, and lipid classes were identified by comparison with lipid standards. Contents of each lipid class were evaluated densitometrically.

Statistics

Data are expressed as means ± SD and were analyzed using Student's t-test. Differences were considered significant at $P < 0.05$.

RESULTS

Synthesis of the six standard SQ-OOH isomers

Figure 2A shows a typical total ion current chromatogram after photooxidized SQ was subjected to LC-CL-MS using an ODS column. After photooxidation, SQ was completely oxidized, giving three peak components (**a–c**) as
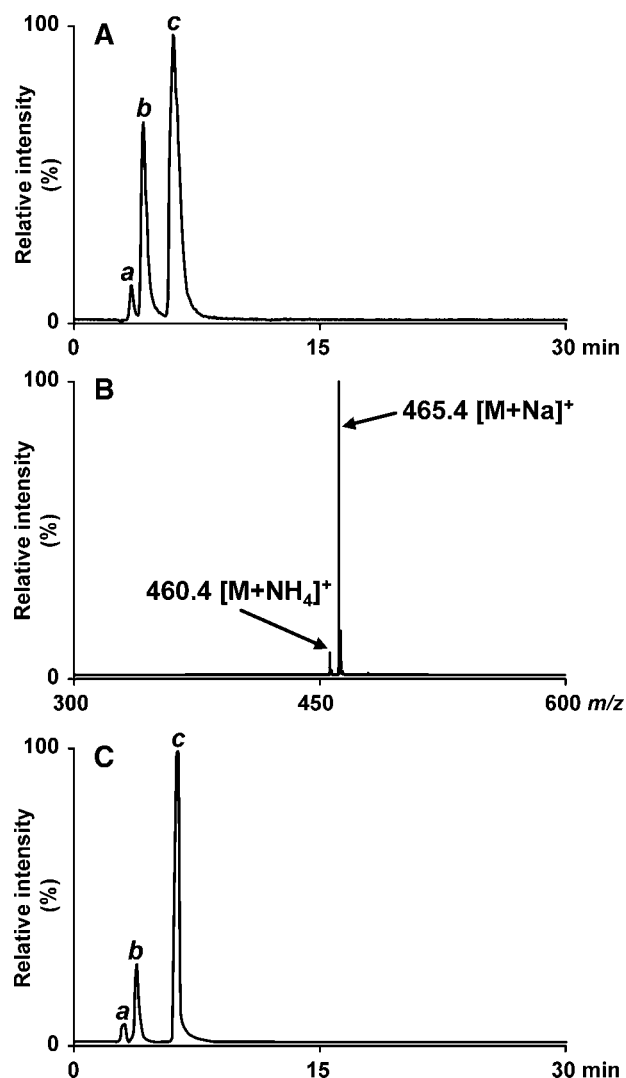
Fig. 2. Photooxidation products of SQ. SQ [100 mg (240 μmol)] was dissolved in 50 ml of ethanol (containing 0.01 mg/ml rose bengal as a sensitizer), photooxidized with a 100 W tungsten lamp at 4°C for 6 h, and analyzed by liquid chromatography-chemiluminescence detection-MS. A: Total ion current chromatogram. B: Mass spectrum of the peak **c** (5.9 min) component in chromatogram A. C: Chemiluminescence chromatogram.

the main oxidation products on the total ion current chromatogram. Peak **c** (retention time, 5.9 min) gave a clear ESI/MS spectrum (m/z 460.4 $[M+NH_4]^+$ and m/z 465.4 $[M+Na]^+$) identical to that of SQ-OOH (Fig. 2B). Similarly, peak components **a** (retention time, 3.4 min) and **b** (retention time, 4.1 min) were identified as SQ-trihydroperoxide (m/z 524.4 $[M+NH_4]^+$ and m/z 529.4 $[M+Na]^+$) and SQ-dihydroperoxide (m/z 492.4 $[M+NH_4]^+$ and m/z 497.4 $[M+Na]^+$), respectively (data not shown). In the CL chromatogram, the peak components **a–c** all gave intense chemiluminescence peaks (Fig. 2C), confirming the presence of hydroperoxide groups.

Because peak component **c** was thought to be a mixture of SQ-OOH isomers (Fig. 1), peak **c** was isolated by semipreparative LC-UV using an ODS column (**Fig. 3A**) and then subjected to LC-UV using a silica column (Fig. 3B). As anticipated, six peak components (**1–6**) were visible in the UV chromatogram. After individual components of peaks **1–6** were isolated, chemical structures were elucidated as SQ-OOH isomers (i.e., peak **1**, 11-OOH-SQ; peak **2**, 7-OOH-SQ; peak **3**, 10-OOH-SQ; peak **4**, 6-OOH-SQ; peak **5**, 3-OOH-SQ; and peak **6**, 2-OOH-SQ; Fig. 1) by NMR (**Table 1**, **Fig. 4**). As judged from NMR spectra, individual SQ-OOH isomers were essentially pure (>98%) and did not contain SQ or other compounds. The pre-

pared SQ-OOH isomers were used as reference standards in later experiments.

LC-MS/MS with MRM analysis of standard SQ-OOH isomers

MS/MS analysis of standard SQ-OOH isomers was first investigated using QTRAP with flow injection. Interestingly, SQ-OOH isomers bearing hydroperoxides at the tertiary carbon atom position (2-OOH-SQ, 6-OOH-SQ, and 10-OOH-SQ) showed intense ions at m/z 425.4 $[M+H-H_2O]^+$ in Q1 mass spectra (**Fig. 5A**), whereas SQ-OOH isomers bearing hydroperoxides at secondary carbon atoms (3-OOH-SQ, 7-OOH-SQ, and 11-OOH-SQ) revealed ions at m/z 425.4 $[M+H-H_2O]^+$ as well as m/z 443.4 $[M+H]^+$ (Fig. 5B). This difference was useful for the discrimination between SQs bearing hydroperoxides at the secondary and tertiary carbon atom positions.

Therefore, we conducted product ion scanning for m/z 425.4 $[M+H-H_2O]^+$ for 2-OOH-SQ, 6-OOH-SQ, and 10-OOH-SQ (**Fig. 6A**) and for m/z 443.4 $[M+H]^+$ for 3-OOH-SQ, 7-OOH-SQ, and 11-OOH-SQ (Fig. 6B) using Q3 in QqLIT mode. Specific fragment ions for each individual SQ-OOH isomer were selected from the various fragment ions observed (**Table 2**). For example, the intensity of fragment ions of 2-OOH-SQ was compared with that of 6-OOH-SQ and 10-OOH-SQ, which identified the 2-OOH-SQ-specific fragment ions as m/z 383.4, 369.4, 355.4, and 341.4 (**Fig. 7**).

The selected fragment ions (Table 2, Fig. 7) allowed the selective detection of SQ-OOH isomers using LC-MS/MS with MRM. In MRM chromatograms (**Fig. 8A**), each SQ-OOH isomer was clearly separated and detected as a single peak. All calibration curves showed good linearity ($R^2 = 0.999$) (Fig. 8B), and detection limits were ∼0.05 ng (0.1 pmol)/injection at a signal-to-noise ratio of 3.

SQ-OOH accumulation in sunlight-exposed human skin

Skin surface lipids were collected from human foreheads before and after 3 h of sunlight exposure using acetone-wet cotton pads. TLC analysis showed that skin surface lipids before sunlight exposure consisted mainly of wax esters (47.8 ± 4.8%), triacylglycerol (22.8 ± 1.9%), and SQ (16.3 ± 1.3%). After exposure, SQ composition decreased to 11.4 ± 0.9% (**Table 3**), which indicated that sunlight exposure caused peroxidation and degradation of skin SQ.

In MRM chromatograms of skin surface lipids before sunlight exposure, all SQ-OOH isomers appeared as clear peaks (**Fig. 9**). Concentrations of SQ-OOH isomers were in the following order: 6-OOH-SQ > 11-OOH-SQ > 2-OOH-SQ > 3-OOH-SQ > 10-OOH-SQ > 7-OOH-SQ (**Fig. 10**). After 3 h of sunlight exposure, concentrations of SQ-OOH isomers were increased markedly.

Finally, to determine the recovery of SQ-OOH, standard 11-OOH-SQ (1–5 μg) was spiked with 5 mg of the skin surface lipid sample and 11-OOH-SQ was extracted and quantified using our LC-MS/MS method. The recovery rate of 11-OOH-SQ was calculated as >90%. The high reproducibility for determining skin surface SQ-OOH (coefficient of
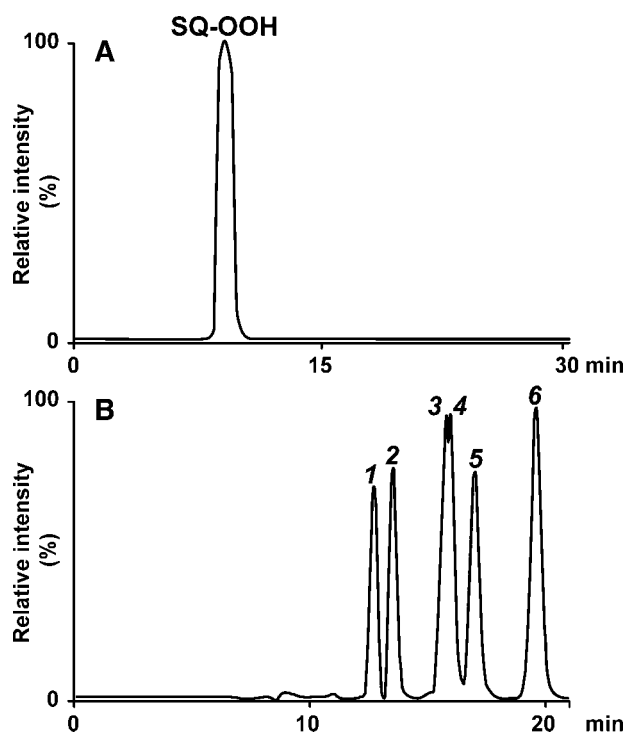

Fig. 3. Purification of SQ-OOH isomers. A: An ethanol solution (3 ml) containing SQ-OOH obtained by rose bengal-catalyzed SQ photooxidation was subjected to semipreparative LC-ultraviolet (UV) (210 nm) using an ODS column, and the SQ-OOH peak (7.9 min; a mixture of SQ-OOH isomers) was isolated. B: The obtained SQ-OOH was evaporated to dryness and redissolved in 1 ml of cyclohexane. The solution was further subjected to LC-UV (210 nm) using a silica column to isolate each of the six SQ-OOH isomers (peaks **1–6**).

TABLE 1. ^{1}H and ^{13}C NMR data of SQ-OOH isomers (peaks **1–6**)

Assignment	11-OOH-SQ (peak *1*)		7-OOH-SQ (peak *2*)		10-OOH-SQ (peak *3*)		6-OOH-SQ (peak *4*)		3-OOH-SQ (peak *5*)		2-OOH-SQ (peak *6*)	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1	1.68 d	25.7	1.68 d	25.7	1.68 d	25.7	1.68 d	25.7	5.01-5.03 br s	144.4	1.34 s	24.2
2	—	131.3	—	132.5	—	131.3	—	131.3	—	143.6	—	82.3
3	5.10 m	124.9	5.17 m	124.4	5.13 m	124.3	5.11 m	124.2	4.29 m	89.2	5.54 m	134.3
4	2.06 m	26.7	2.20 m	26.3	2.07 m	26.4	2.00 m	39.6	1.57-1.60 m	29.0	5.68 m	136.4
5	1.98 m	39.7	2.06-2.13 m	30.8	2.00 m	39.6	2.00 m	39.6	1.97-2.01 m	35.1	2.72 m	42.6
6	—	136.1	—	147.4	—	135.1	—	84.5	—	134.9	—	135.1
7	5.10 m	124.1	4.34 m	89.2	5.09 m	121.3	5.48 m	132.1	5.11 m	124.2	5.11 m	125.5
8	2.22 m	26.2	1.60-1.68 m	29.6	2.16 m	26.4	5.66 m	131.3	2.08 m	26.6	2.08 m	28.2
9	2.06-2.12 m	31.0	2.06-2.60 m	35.7	1.61 m	37.6	2.78 m	31.0	1.96 m	29.7	1.98 m	39.6
10	—	147.4	—	135.3	—	84.5	—	135.3	—	133.9	—	134.9
11	4.34 m	89.0	5.17 m	125.0	5.47 m	132.1	5.11 m	124.2	5.14 m	124.3	5.11 m	124.4
12	1.53-1.63 m	31.5	2.05 m	28.3	5.50 m	131.2	2.02 m	28.3	1.91 m	28.2	1.98 m	26.6
13	2.05 m	24.2	2.05 m	28.2	2.70 m	31.0	1.98 m	39.7	2.07 m	39.7	2.02 m	39.6
14	5.12 m	123.3	5.11 m	124.1	5.11 m	124.3	5.11 m	125.6	5.13 m	124.3	5.11 m	124.3
15	—	135.0	—	134.9	—	136.7	—	134.9	—	134.5	—	134.9
16	1.98 m	26.6	2.17 m	26.6	2.02 m	26.7	2.02 m	26.7	1.97 m	26.6	1.98 m	26.6
17	2.06 m	39.7	1.90 m	39.7	2.08 m	39.6	2.07 m	39.7	2.04 m	39.7	2.08 m	39.7
18	5.16 m	123.9	5.12 m	124.2	5.11 m	124.2	5.11 m	124.2	5.09 m	124.2	5.11 m	124.2
19	—	136.0	—	134.2	—	134.7	—	131.9	—	135.1	—	133.2
20	1.98 m	26.6	2.09 m	26.8	2.00 m	20.4	2.00 m	22.5	2.01 m	26.5	2.00 m	26.7
21	2.06 m	39.7	1.98 m	39.7	1.66 m	39.6	1.60 m	39.7	2.07 m	39.7	1.68 m	39.7
22	5.10 m	124.2	5.12 m	124.2	5.15 m	124.0	5.11 m	124.2	5.08 m	125.0	5.10 m	124.2
23	—	131.3	—	131.3	—	131.2	—	131.3	—	131.2	—	124.3
24	1.68 d	25.7	1.68 d	25.7	1.68 d	25.7	1.68 d	25.7	1.68 d	25.7	1.68 d	24.2
25	1.60 d	25.7	1.60 d	17.8	1.60 d	17.6	1.60 d	21.5	1.70 d	17.1	1.34 s	16.0
26	1.60 d	16.1	5.06-5.13 br s	113.2	1.60 d	16.1	1.60 d	21.5	1.60 d	14.0	1.58 d	16.0
27	5.06-5.11 br s	113.2	1.60 d	16.0	1.33 s	21.5	1.33 s	21.5	1.60 d	16.0	1.60 d	16.0
28	1.60 d	17.0	1.60 d	16.0	1.60 d	17.0	1.60 d	16.0	1.60 d	16.0	1.60 d	16.0
29	1.60 d	16.1	1.60 d	16.1	1.60 d	16.1	1.60 d	16.0	1.60 d	15.9	1.60 d	16.0
30	1.60 d	17.7	1.60 d	17.7	1.60 d	17.7	1.60 d	17.0	1.60 d	17.6	1.60 d	17.6
-OOH	7.84 s		7.86 s		7.24 s		7.22 s		7.76 s		7.27 s	

variability < 5%) was confirmed and was not altered by the storage of skin surface lipid samples at −80°C for 1 week. These results indicated the quantitative accuracy of the LC-MS/MS method. On the other hand, the total concen-tration of skin surface SQ-OOH isomers determined by LC-MS/MS (before sunlight exposure, 956 ± 496 μg/g skin lipids; after 3 h of exposure, 2,764 ± 1,130 μg/g skin lipids) was similar to the results obtained by LC-CL (before sun-light exposure, 611 ± 221 μg/g skin lipids; after 3 h, 1,877 ± 996 μg/g skin lipids).
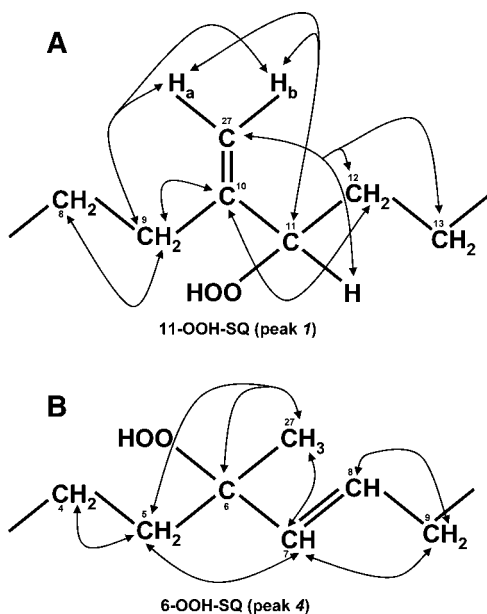

Fig. 4. Representative heteronuclear multiple bond correlation for two different partial structures of SQ-OOH (A, peak **1**, 11-OOH-SQ; B, peak **4**, 6-OOH-SQ).

DISCUSSION

The increasing evidence for the involvement of skin lipid peroxidation in inflammatory skin disorders (28, 29) has shown the need for analytical methods to detect SQ-OOH isomers. However, a method for the characteriza-tion and quantitation of SQ-OOH isomers in vivo had not yet been established. Therefore, the objectives of the present study were to develop a preparation method of reliable standard SQ-OOH isomers, as well as an analytical method applicable to biological samples, and then to de-termine the occurrence of SQ-OOH isomers in vivo.

The lack of commercially available or suitable hydroper-oxide standards was a major problem for the measurement of lipid hydroperoxides. Lipid hydroperoxides prepared from photooxidized or free radical-oxidized lipids are gen-erally used as standard compounds (30). However, the purity of hydroperoxides in such standards is not generally high. Impurities in standards can be attributable to ana-lytical errors. To develop analytical methods for SQ-OOH isomers, pure standards are essential. Therefore, to gener-
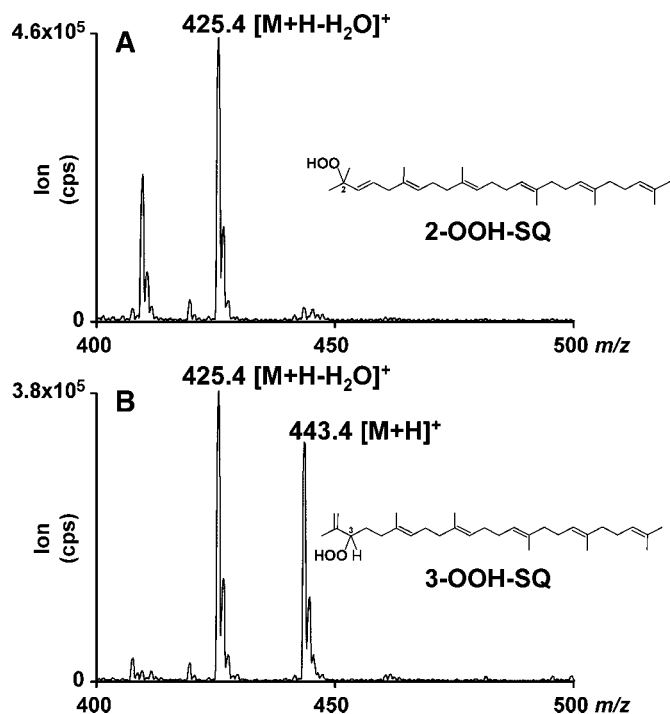
Fig. 5. Typical Q1 mass spectra of SQ bearing a hydroperoxide at the tertiary carbon atom position (2-OOH-SQ) (A) or at the secondary carbon atom position (3-OOH-SQ) (B). Standard solutions of 2-OOH-SQ or 3-OOH-SQ [each 0.4 μg (1 nmol)/ml] were infused directly into the QTRAP MS/MS instrument using a syringe pump at a flow rate of 0.01 ml/min.

ate SQ-OOH, a photooxidation reaction in the presence of rose bengal as a catalyst was chosen, because a reaction that generated singlet oxygen as an emitter was considered preferable. As a result, six SQ-OOH isomers (11-OOH-SQ, 7-OOH-SQ, 10-OOH-SQ, 6-OOH-SQ, 3-OOH-SQ, and 2-OOH-SQ) were generated as the predominant products (Figs. 2, 3). 2-OOH-SQ, 6-OOH-SQ, and 10-OOH-SQ were presumably racemic mixtures. It is interesting that no peroxidation was detected at C-4, C-8, or C-12 of SQ. Six SQ-OOH isomers were then separated and isolated by LC techniques and provided standard SQ-OOH with high purity (>98%) (Fig. 3) and stability for at least 3 months in 1-butanol at −80°C. On the other hand, according to ^{1}H NMR analysis of each SQ-OOH isomer, a characteristic signal was observed at around δ 7.0–8.0. The signal was shifted downfield by 3 ppm when SQ-OOH was dissolved in acetone-d_6 (instead of CDCl$_3$). The signal disappeared when deuterated water was added to the SQ-OOH dissolved in acetone-d_6. These features were characteristic for the proton of the hydroperoxide group. As shown in Fig. 4, structural differences around the hydroperoxide group could be assigned mainly by heteronuclear multiple bond correlation compared with the known chemical shift of SQ-OOH (16).

After MS/MS analysis of the synthesized SQ-OOH standards, we discovered significant differences in molecular ion intensity between SQ isomers bearing hydroperoxides at tertiary carbon atoms (2-OOH-SQ, 6-OOH-SQ, and 10-OOH-SQ) and secondary carbon atoms (3-OOH-SQ, 7-
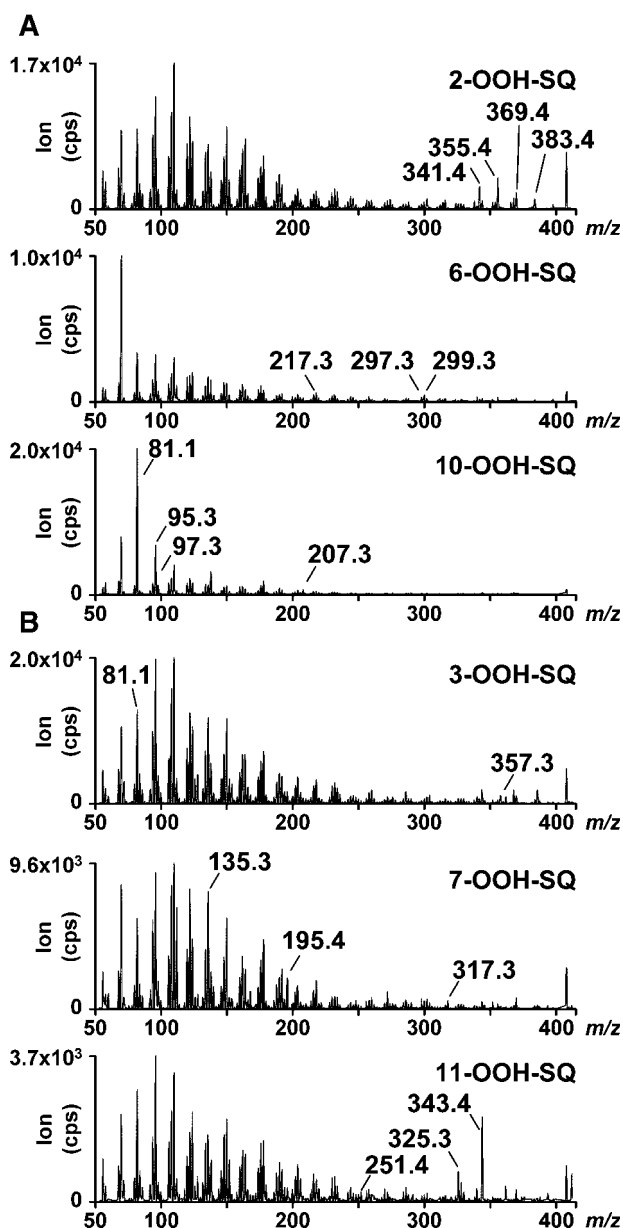

Fig. 6. Typical Q3 mass spectra (product ion scanning) for m/z 425.4 [M+H-H$_2$O]$^+$ for 2-OOH-SQ, 6-OOH-SQ, and 10-OOH-SQ (A) and for m/z 443.4 [M+H]$^+$ for 3-OOH-SQ, 7-OOH-SQ, and 11-OOH-SQ (B) using Q3 in the hybrid quadrupole/linear ion trap mode. Each standard SQ-OOH isomer [0.4 μg (1 nmol)/ml] was infused directly into the QTRAP MS/MS instrument using a syringe pump at a flow rate of 0.01 ml/min.

OOH-SQ, and 11-OOH-SQ) (Fig. 5). Based on this finding, we selected specific fragment ions for each individual SQ-OOH isomer (Table 2, Figs. 6, 7), which permitted the selective detection of SQ-OOH isomers using LC-MS/MS with MRM (Fig. 8). A silica column was used, as such columns have been used successfully in past studies for the separation of positional isomers of organic hydroperoxides (31). Under optimized conditions, standard SQ-OOH isomers could be clearly separated and detected on MRM chromatograms. The resultant calibration curves were linear over a wide concentration range, which allowed the calculation

TABLE 2. Specific fragment ions for each SQ-OOH isomer

Isomer	Peak Number	Precursor Ion	Specific Fragment Ions	Multiple Reaction Monitoring	Retention Time
					min
2-OOH-SQ	6	425.4	341.4, 355.4, 369.4, 383.4	425.4/341.4	7.3
3-OOH-SQ	5	443.4	81.1, 357.3	4434./81.1	5.8
6-OOH-SQ	4	425.4	217.3, 297.3, 299.3	425.4/297.3	5.7
7-OOH-SQ	2	443.4	135.3, 195.4, 317.3	443.4/195.4	4.5
10-OOH-SQ	3	425.4	81.1, 95.3, 97.3, 207.3	425.4/81.1	5.5
11-OOH-SQ	1	443.4	251.4, 325.3, 343.4	443.4/343.4	4.0

of corresponding endogenous levels of SQ-OOH isomers. The LC-MS/MS with MRM method provided almost 10-fold greater sensitivity [detection limit, 0.05 ng (0.1 pmol) SQ-OOH/injection] compared with the LC-CL method (7, 8). The sensitivity of the present LC-MS/MS with MRM method for SQ-OOH was relatively high, as LC-CL detection limits for lipid hydroperoxides, such as phospholipid hydroperoxides (5, 6) and cholesterol hydroperoxides (22, 32), are generally above the picomole range.

Normally, it is very difficult to detect SQ-OOH in biological samples because of the interference of background contaminants, even when using LC-CL. In the present study, the use of LC-MS/MS with MRM allowed individual SQ-OOH isomers in skin surface lipid extracts from human forehead to be structurally identified and quantitated (Fig. 9). Before sunlight exposure, the relative amount of skin surface SQ-OOH to SQ was ~1.09 mol% (Figs. 9, 10), such that small amounts of SQ had peroxidized and con-

verted to SQ-OOH under daily life conditions. As highly peroxidized products such as SQ-dihydroperoxide and SQ-trihydroperoxide were undetectable in human skin (data not shown), sunlight stress may not be a powerful factor in terms of daily life conditions. After 3 h of sunlight exposure, the accumulation of skin surface SQ-OOH isomers was observed. Given our results and previous reports (7, 8), it appears that skin SQ-OOH level would be a useful marker for sunlight exposure. On the other hand, percentages of SQ-OOH isomers after 3 h of sunlight exposure (17 mol% 2-OOH-SQ, 14 mol% 3-OOH-SQ, 24 mol% 6-OOH-SQ, 11 mol% 7-OOH-SQ, 13 mol% 10-OOH-SQ, and 21 mol% 11-OOH-SQ) were similar to those found in 6 h rose bengal-catalyzed photooxidation of SQ (19 mol%
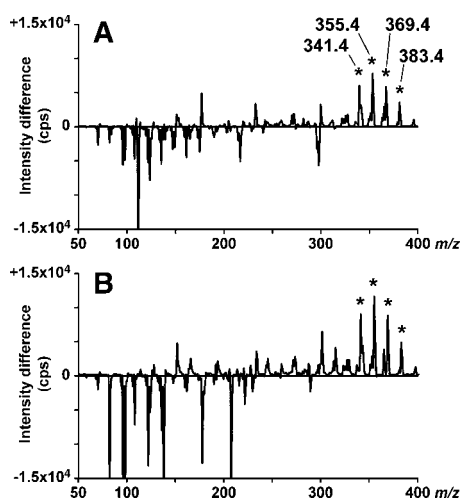

Fig. 7. Differences of fragment ion intensities between 2-OOH-SQ and 6-OOH-SQ (A) and between 2-OOH-SQ and 10-OOH-SQ (B). Because the detected fragment ion intensities varied among these SQ-OOH isomers (e.g., see the *y* axis of Fig. 6A, 1.0×10^4 to 2.0×10^4 cps), the following calculation was performed: A/(C/D) − B, where A is the fragment ion intensity of 2-OOH-SQ at a specific *m/z*, B is the fragment ion intensity of 6-OOH-SQ at the same specific *m/z*, C is the total accumulated ions of 2-OOH-SQ (1.2×10^6 cps) between *m/z* 50 and 415, and D is the total accumulated ions of 6-OOH-SQ (4.2×10^5 cps) in the same *m/z* range. Similarly, the difference of fragment ion intensity between 2-OOH-SQ and 10-OOH-SQ was calculated. Based on the results shown, the 2-OOH-SQ-specific fragment ions were identified as *m/z* 383.4, 369.4, 355.4, and 341.4. These ions are labeled with asterisks.
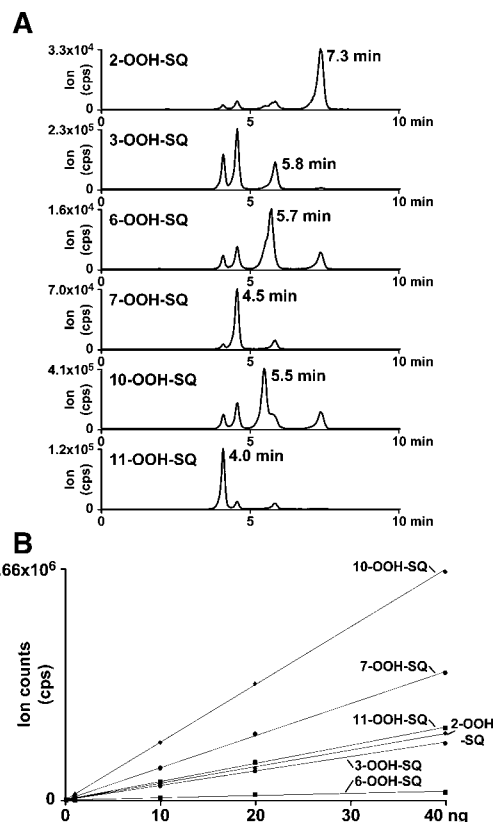

Fig. 8. A: Typical multiple reaction monitoring (MRM) chromatograms of standard SQ-OOH isomers. A mixture of six standard SQ-OOH isomers (2-OOH-SQ, 3-OOH-SQ, 6-OOH-SQ, 7-OOH-SQ, 10-OOH-SQ, and 11-OOH-SQ; each 10 ng) was analyzed by LC-MS/MS with MRM. B: Calibration curves of SQ-OOH isomers constructed using different concentrations of standards (0.1–40 ng/injection).

TABLE 3. Composition of skin surface lipids before and 3 h after sunlight exposure

Constituent	Before Sunlight Exposure	After Sunlight Exposure
	weight %	
SQ	16.3 ± 1.3	11.4 ± 0.9
Free fatty acid	5.0 ± 0.7	6.2 ± 1.3
Wax ester	47.8 ± 4.8	48.4 ± 4.5
Triacylglycerol	22.8 ± 1.9	23.4 ± 2.1
Cholesteryl ester	5.6 ± 2.7	8.4 ± 2.7
Cholesterol	2.8 ± 1.8	2.4 ± 1.1

Values are means ± SD.

2-OOH-SQ, 17 mol% 3-OOH-SQ, 24 mol% 6-OOH-SQ, 10 mol% 7-OOH-SQ, 13 mol% 10-OOH-SQ, and 17 mol% 11-OOH-SQ). Therefore, singlet oxygen derived from sunlight would cause skin SQ peroxidation, and "2-OOH-SQ > 3-OOH-SQ" and "6-OOH-SQ > 7-OOH-SQ" may be explained by the greater stability of tertiary/allylic radicals. However, another oxidant (e.g., ozone) than singlet oxygen may also attack on skin SQ, and the reason for "11-OOH-SQ > 10-OOH-SQ" was unknown. Further in-
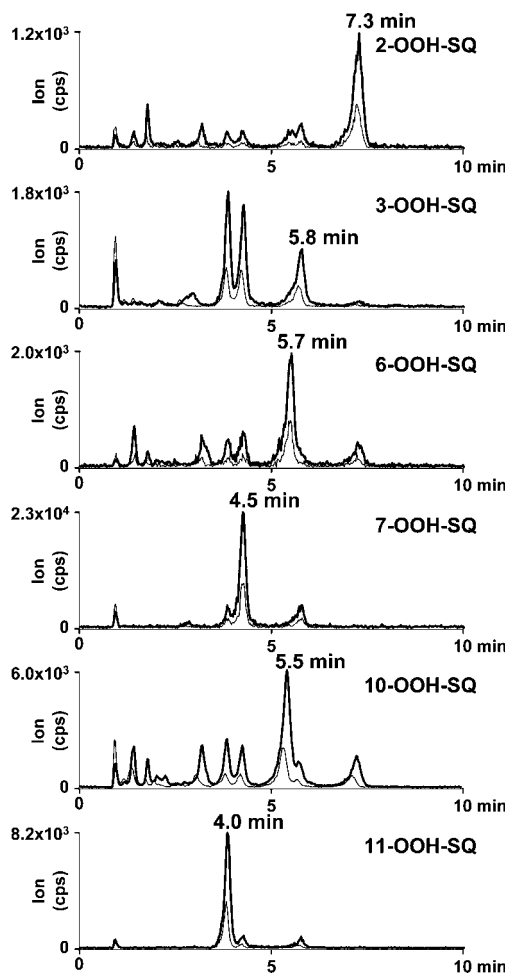

Fig. 9. Typical MRM chromatograms of skin surface lipids before (thin lines) and after (thick lines) 3 h of sunlight exposure. Human forehead skin lipid extract (10 µl) was subjected to LC-MS/MS with MRM.
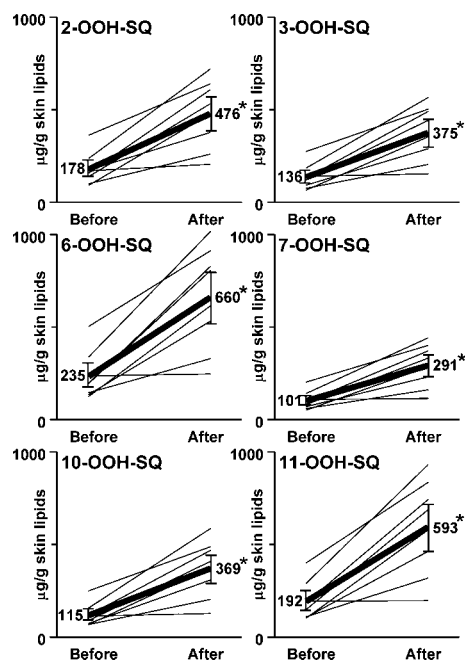

Fig. 10. Concentrations of skin surface SQ-OOH isomers before and after 3 h of sunlight exposure. Human forehead skin lipid extracts (10 µl) were subjected to LC-MS/MS with MRM. Values are means ± SD. * Significant difference compared with before sunlight exposure.

vestigation will be required to evaluate the peroxidation mechanism of skin SQ.

The human skin surface, constantly exposed to environmental stress, is thought to be vulnerable to attack from singlet oxygen generated by sunlight exposure (33). Some recent studies have reported that SQ-OOH is cytotoxic and increases the secretion of various inflammatory cytokines by human keratinocytes in a three-dimensional human skin model (34). SQ-OOH also enhanced NF-kB nuclear translocation, followed by increased expression and secretion of the proinflammatory cytokine IL-6 (35). Therefore, it is likely that excessive SQ peroxidation affects the maintenance of cell integrity and survival, contributing to pathogenesis. Sies and Stahl (36) reviewed the interaction between oxidative stress and the antioxidative network of skin. Therefore, the development of inhibitors of SQ peroxidation may provide a therapeutic approach for skin disorders. On the other hand, UV-induced DNA damage is a critical event in skin diseases, especially cancer (37). Constitutive skin pigmentation affects the incidence of skin cancer, and the photoprotective function of melanin in the skin is important (37). Thus, we are currently investigating the effect of SQ peroxidation on skin DNA and pigments.

Recently, Mountfort et al. (38) investigated oxidation products of SQ in latent fingerprints using LC-MS and confirmed that SQ-OOH and SQ-epoxide were formed within 1 day when prints were exposed to light. Considering those study results (38) and the present findings (Fig. 10), SQ oxidation products, especially 6-OOH-SQ, 11-OOH-SQ, and 2-OOH-SQ, may become suitable targets in forensic science.

In conclusion, we developed a QTRAP LC-MS/MS method for determining SQ-OOH isomers in human skin surface lipids. Our findings provide the first structural and quantitative evidence for the peroxidation of skin SQ occurring during exposure to sunlight. To further understand the involvement of SQ peroxidation in the pathophysiology of skin disease, we are now using our LC-MS/MS method to quantify SQ-OOH isomers in UV-exposed human sebum. Based on our method, it is now feasible to develop new analytical techniques for the determination of skin lipid peroxidation products other than SQ-OOH.⬛

REFERENCES

1. Bruls, W. A., H. Van Weedlden, and J. C. Van der Leun. 1984. Transmission of UV-radiation through human epidermal layers as a factor influencing the minimal erythema dose. *Photochem. Photobiol.* **39:** 63–67.
2. Chew, S., V. A. DeLeo, and L. C. Harber. 1987. An animal model for evaluation of topical photoprotection against ultraviolet A (320–380 nm) radiation. *J. Invest. Dermatol.* **89:** 410–414.
3. Fujita, H., I. Matsuo, M. Okazaki, K. Yoshino, and M. Ohkido. 1986. Chlorpromazine-sensitized photooxidation of squalene. *Arch. Dermatol. Res.* **278:** 224–227.
4. Ramasastry, P., D. T. Downing, P. E. Pochi, and J. S. Strauss. 1970. Chemical composition of human skin surface lipids from birth to puberty. *J. Invest. Dermatol.* **54:** 139–144.
5. Miyazawa, T. 1989. Determination of phospholipid hydroperoxides in human blood plasma by a chemiluminescence-HPLC assay. *Free Radic. Biol. Med.* **7:** 209–217.
6. Kinoshita, M., S. Oikawa, K. Hayasaka, A. Sekikawa, T. Nagashima, T. Toyota, and T. Miyazawa. 2000. Age-related increase in plasma phosphatidylcholine hydroperoxide concentrations in normal subjects and patients with hyperlipidemia. *Clin. Chem.* **46:** 822–828.
7. Kohno, Y., O. Sakamoto, K. Tomita, I. Horii, and T. Miyazawa. 1991. Determination of human skin surface lipid peroxides by chemiluminescence-HPLC. *J. Jpn. Oil Chem. Soc.* **40:** 715–718.
8. Kohno, Y., O. Sakamoto, T. Nakamura, and T. Miyazawa. 1993. Determination of human skin surface lipid peroxides by chemiluminescence-HPLC. II. Detection of squalene hydroperoxide. *J. Jpn. Oil Chem. Soc.* **42:** 204–209.
9. Langdon, R. G., and K. Bloch. 1953. The utilization of squalene in the biosynthesis of cholesterol. *J. Biol. Chem.* **200:** 135–144.
10. Kohno, Y., Y. Egawa, S. Itoh, S. Nagaoka, M. Takahashi, and K. Mukai. 1995. Kinetic study of quenching reaction of singlet oxygen and scavenging reaction of free radical by squalene in n-butanol. *Biochim. Biophys. Acta.* **1256:** 52–56.
11. Kohno, Y., and M. Takahashi. 1995. Peroxidation in human skin and its prevention. *J. Jpn. Oil. Chem. Soc.* **44:** 10–17.
12. Dennis, K. J., and T. Shibamoto. 1989. Production of malonaldehyde from squalene, a major skin surface lipid, during UV-irradiation. *Photochem. Photobiol.* **49:** 711–716.
13. Maes, D., T. Mammone, M. A. McKeever, E. Pelle, C. Fthenakis, L. Declercq, P. U. Giacomoni, and K. Marenus. 2000. Noninvasive techniques for measuring oxidation products on the surface of human skin. *Methods Enzymol.* **319:** 612–622.
14. Ohsawa, K., T. Watanabe, R. Matsukawa, Y. Yoshimura, and K. Imaeda. 1984. The possible role of squalene and its peroxide of the sebum in the occurrence of sunburn and protection from the damage caused by UV irradiation. *J. Toxicol. Sci.* **9:** 151–159.
15. Chiba, K., K. Yoshizawa, I. Makino, K. Kawakami, and M. Onoue. 2000. Comedogenicity of squalene monohydroperoxide in the skin after topical application. *J. Toxicol. Sci.* **25:** 77–83.
16. Mudiyanselage, S. E., M. Hamburger, P. Elsner, and J. J. Thiele. 2003. Ultraviolet A induces generation of squalene monohydroperoxide isomers in human sebum and skin surface lipids in vitro and in vivo. *J. Invest. Dermatol.* **120:** 915–922.
17. Migliorança, L. H., R. E. Barrientos-Astigarraga, B. S. Schug, H. H. Blume, A. S. Pereira, and G. D. Nucci. 2005. Felodipine quantification in human plasma by high-performance liquid chromatography coupled to tandem mass spectrometry Qtrap. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **814:** 217–223.
18. Yao, M., H. Zhang, S. Chong, M. Zhu, and R. A. Morrison. 2003. A rapid and sensitive LC/MS/MS assay for quantitative determination of digoxin in rat plasma. *J. Pharm. Biomed. Anal.* **32:** 1189–1197.
19. Houjou, T., K. Yamatani, H. Nakanishi, M. Imagawa, T. Shimizu, and R. Taguchi. 2004. Rapid and selective identification of molecular species in phosphatidylcholine and sphingomyelin by conditional neutral loss scanning and MS3. *Rapid Commun. Mass Spectrom.* **18:** 3123–3130.
20. Nakagawa, K., J. H. Oak, O. Higuchi, T. Tsuzuki, S. Oikawa, H. Otani, M. Mune, H. Cai, and T. Miyazawa. 2005. Ion-trap tandem mass spectrometric analysis of Amadori-glycated phosphatidylethanolamine in human plasma with or without diabetes. *J. Lipid Res.* **46:** 2514–2524.
21. Gandin, E., Y. Lion, and V. D. Vorst. 1983. Quantum yield of singlet oxygen production by xanthene derivatives. *Photochem. Photobiol.* **37:** 271–278.
22. Tagiri-Endo, M., K. Nakagawa, T. Sugawara, K. Ono, and T. Miyazawa. 2004. Ozonation of cholesterol in the presence of ethanol: identification of a cytotoxic ethoxyhydroperoxide molecule. *Lipids.* **39:** 259–264.
23. Miyazawa, T., K. Yasuda, and K. Fujimoto. 1987. Chemiluminescence-high performance liquid chromatography of phosphatidylcholine hydroperoxide. *Anal. Lett.* **20:** 915–925.
24. Miyazawa, T., T. Suzuki, K. Fujimoto, and K. Yasuda. 1992. Chemiluminescent simultaneous determination of phosphatidylcholine hydroperoxide and phosphatidylethanolamine hydroperoxide in the liver and brain of the rat. *J. Lipid Res.* **33:** 1051–1059.
25. Marzouki, Z. M., A. M. Taha, and K. S. Gomma. 1987. Variability in the composition of human skin surface lipids in tropical climates. *Trop. Geogr. Med.* **39:** 366–371.
26. Haze, S., Y. Gozu, S. Nakamura, Y. Kohno, K. Sawano, H. Ohta, and K. Yamazaki. 2001. 2-Nonenal newly found in human body odor tends to increase with aging. *J. Invest. Dermatol.* **116:** 520–524.
27. Downing, D. T. 1968. Photodensitometry in the thin-layer chromatographic analysis of neutral lipids. *J. Chromatogr.* **38:** 91–99.
28. Chiba, K., K. Kawakami, T. Sone, and M. Onoue. 2003. Characteristics of skin wrinkling and dermal changes induced by repeated application of squalene monohydroperoxide to hairless mouse skin. *Skin Pharmacol. Appl. Skin Physiol.* **16:** 242–251.
29. Rho, H. S., H. S. Baek, B. S. Lee, J. H. Kim, D. H. Kim, and I. S. Chang. 2006. New hydroxamic acid derivative prevents sq-OOH induced wrinkle. *Bull. Korean Chem. Soc.* **27:** 115–118.
30. Hui, S-P., T. Yoshimura, T. Murai, H. Chiba, and T. Kurosawa. 2000. Determination of regioisomeric hydroperoxides of fatty acid cholesterol esters produced by photosensitized peroxidation using HPLC. *Anal. Sci.* **16:** 1023–1028.
31. Melchior, D., and S. Gab. 2000. Simultaneous micellar electrokinetic chromatographic determination of isomeric fatty acid hydroperoxides and corresponding hydroxy fatty acids. *J. Chromatogr. A.* **894:** 145–155.
32. Adachi, J., M. Asano, T. Naitob, Y. Ueno, and Y. Tatsuno. 1998. Chemiluminescent determination of cholesterol hydroperoxides in human erythrocyte membrane. *Lipids.* **33:** 1235–1240.
33. Urbac, F. 1989. Potential effects of altered solar ultraviolet radiation on human skin cancer. *Photochem. Photobiol.* **50:** 507–513.
34. Uchino, T., H. Tokunaga, H. Onodera, and M. Ando. 2002. Effect of squalene monohydroperoxide on cytotoxicity and cytokine release in a three-dimensional human skin model and human. *Biol. Pharm. Bull.* **25:** 605–610.
35. Ottaviani, M., T. Alestas, E. Flori1, A. Mastrofrancesco1, C. Zouboulis, and M. Picardo. 2006. Peroxidated squalene induces the production of inflammatory mediators in HaCaT keratinocytes: a possible role in acne vulgaris. *J. Invest. Dermatol.* **126:** 2430–2437.
36. Sies, H., and W. Stahl. 2004. Nutritional protection against skin damage from sunlight. *Annu. Rev. Nutr.* **24:** 173–200.
37. Tadokoro, T., N. Kobayashi, B. Z. Zmudzka, S. Ito, K. Wakamatsu, Y. Yamaguchi, K. S. Korossy, S. A. Miller, J. Z. Beer, and V. J. Hearing. 2003. UV-induced DNA damage and melanin content in human skin differing in racial/ethnic origin. *FASEB J.* **17:** 1177–1179.
38. Mountfort, K. A., H. Bronstein, N. Archer, and S. M. Jickells. 2007. Identification of oxidation products of squalene in solution and in latent fingerprints by ESI-MS and LC/APCI-MS. *Anal. Chem.* **79:** 2650–2657.

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