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A systematic method for the sensitive and specific determination of hair lipids in combination with chromatography

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Abstract

A systematic method for the sensitive, precise and accurate determination of hair lipids, including trace amounts of intrinsic endogenous cholesterol (CH), ceramide/*N*-palmitoyl-DL-dihydrosphingosine (CER/PDS), cholesterol sulfate (CS) and chemically bound 18-methyl eicosanoic acid (18-MEA), has been developed in combination with TLC/FID (flame ionization detection), LC/MS and GC/MS. TLC/FID was used for the simultaneous determination of squalene (SQ), wax esters (WEs), triglycerides (TGs) and free fatty acids (FFAs). Optimal conditions for LC/MS to determine CS and 18-MEA were developed using selected ion monitoring (SIM) under the negative ion mode of electrospray ionization. An alternative procedure for the determination of 18-MEA was also established using commercially available heneicosanoic acid (HEA). In GC/MS, the optimal selection of ions for SIM of trimethylsilylated CH and CER/PDS, and the use of on-column injection has enabled their simultaneous detection. This newly developed method has been used to characterize the hair lipid composition from the proximal root end to the distal tip of chemically untreated hair fibers from two different females, and specific changes of hair lipids probably due to its origin and individuals have been demonstrated for the first time. This method may be useful for clarifying the important roles of intrinsic endogenous 18-MEA, CS, CH and CERs in the function of the cell membrane complex of hair fibers.

Keywords: Hair; Lipids; TLC/FID; GC/MS; LC/MS

1. Introduction

Hair lipids exist in an intercellular space designated as the cell membrane complex (CMC) located between the cuticle or cortical cells [1], as phospholipids derived from living cells or intercellular lipids of the stratum corneum accumulate in bilayer cell membrane or multi-lamellar structure, respectively [2,3]. The hair lipids of the CMC seem to play roles in determining the physicochemical properties of hair fibers, including the chemical diffusion barrier, the water holding property and the cell cohesion [4–7]. Attention has also been paid to hair lipids as diagnostic indicators for lipid abnormality-related diseases [8,9]. Many analytical studies of hair lipids have revealed that there are squalene (SQ), wax esters (WEs), triglycerides (TGs), free fatty acids (FFAs), cholesterol (CH), ceramides (CERs) and cholesterol sulfate (CS) within hair fibers [6,8–20]. Recently, 18-methyl eicosanoic acid (18-MEA) has been discovered, which chemically binds to the cuticle surface by a thioester linkage [21]. Since CH, CERs, CS and 18-MEA are intrinsic endogenous components of human hair [20], their analysis seems important to identify physicochemical functions of the CMC, in spite of their presence in trace amounts.

For analyses of all hair lipids, TLC (HPTLC)/densitometry using silica gel plates has been used conventionally [10–15,17,19,20]. The TLC method has been extremely valuable for determining the major lipids in hair fibers according to their classes since it can separate them based upon their wide polarity due to various alkyl chains. Therefore, the TLC method can simultaneously determine SQ, WEs, TGs and FFAs in hair lipids. However, trace amounts of intrinsic endogenous lipids, such as CH, CERs, CS and 18-MEA, are difficult to analyze because of their poor mutual separation, low sensitivity, and insufficient accuracy and precision in the TLC method. Thus, during TLC analysis of hair lipids,

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we have often noticed that CH overlaps with higher alcohols derived from the hydrolysis of WEs, and also that CERs and CS overlap with trace surfactants that might originate from shampoos or conditioners used in daily hair care. As alternatives for TLC, methods exist using GC/flame ionization detection (FID) and LC/fluorescence detection (FLD) to determine CH, CERs, CS and 18-MEA in hair lipids [6,13,16–18,20,21]. The GC/FID method is superior from the viewpoint of its low interference from other substances in hair fibers, due to its higher separation ability. However, the sensitivity of GC/FID often does not allow us the determination of such trace lipids as CH, CERs, CS and 18-MEA. Although the LC/FLD method is applicable to the sensitive determination of CERs and 18-MEA [20], they are troublesome and time-consuming due to the requirement for complicated pre-treatments such as fluorescent derivatization, especially in the case of CERs. One of the most important issues in conventional methods involves the fact that these intrinsic endogenous lipids must be individually determined using complicated procedures even if sensitive determination is possible. Thus, troublesome and time-consuming procedures are required for each exclusive method for analyzing lipids, which can lead to complications due to artifacts.

Recently, analytical methods not requiring complicated pre-treatments have become available to determine trace amounts of lipids using mass spectrometry [22–25]. Therefore, it might be expected that chromatographic techniques employing mass spectrometry would provide sensitive, precise and accurate determinations of intrinsic endogenous lipids, such as CH, CERs, CS and 18-MEA, in hair fibers. In addition, a method requiring amounts less than 100 mg of hair fibers would also be desirable. Collecting 100 mg of the proximal root region from the scalp of a healthy female is extremely difficult and only the proximal root region must be used to characterize the precise hair lipid composition for an individual, because hair lipids are gradually lost or altered as the hair fibers grow [20].

The aim of this study was to develop a systematic method to determine all hair lipids, including the trace intrinsic endogenous ones, using as small an amount of hair fibers as possible. We have optimized the analytical conditions for the simultaneous determination by (1) TLC/FID for SQ, WEs, TGs and FFAs, by (2) LC/MS for CS and 18-MEA, and by (3) GC/MS for CH and CERs, and we have validated each analytical procedure. Finally, we have applied this method to characterize the hair lipid composition along the distance from the proximal root end to the distal tip of chemically untreated hair fibers that were collected from two females.

2. Experimental

2.1. Chemicals

Squalene, palmityl palmitate, tripalmitin, palmitic acid, cholesterol, β-cholestanol (BC), cholesterol sulfate sodium salt and heneicosanoic acid (HEA) were purchased from Sigma (St. Louis, MO, USA). 18-MEA (purity 96.0% by GC/FID) was synthesized based upon the Wittig reaction between ω-phosphonium fatty acid methyl ester and branched aldehyde [26]. *N*-Palmitoyl-DL-dihydrosphingosine (CER/PDS) and *N*-myristoyl sphingosine (CER/MS) was from Funakoshi (Tokyo, Japan) and Avanti-Polar Lipids (Alabaster, AL, USA), respectively. Stock individual standard solutions (ca. 1 mg/ml) were prepared by dissolving accurate amounts of authentic lipids in chloroform-methanol (1:1, v/v) and were stored at −4 °C. Working authentic lipid mixtures were obtained by further dilution of stock solutions with chloroform-methanol (1:1, v/v).

Hexane, chloroform and methanol were of infinity pure grade from Wako Pure Chemicals Industries (Tokyo, Japan) and were used to prepare the hair lipid samples. Methanol was of HPLC grade from Kanto Reagents (Tokyo, Japan) and was used to prepare the mobile phase solution. Pyridine of infinity pure grade from Wako and *N*-trimethylsilylimidazole (TMSI) from GL Science (Tokyo, Japan) were used for the trimethylsilylation of hair lipids in GC/MS analysis. Ultra-pure water prepared using a Milli-Q purification system (Millipore, Bedford, MA, USA) was used in all procedures. All other chemicals were of the highest analytical grade available.

2.2. Materials

We developed a new analytical method to determine all hair lipids, using the 10 cm-lengths of scalp hair fibers distant from the proximal root end, which were obtained from healthy Japanese female volunteers. In determining the hair lipids in longitudinal regions from the proximal root end to the distal tip, ca. 40 fibers were obtained from two individuals whose hair had never been treated with any chemical reactions, such as coloring or permanent waving. After each hair sample (designated as Hair-A and Hair-B) was cut every 4 cm from the proximal root end, they were washed with plain shampoo for 1 min and rinsed with water for 10 min. This procedure was repeated twice. The hair samples were then dried at room temperature for more than 48 h. After washing with hexane for 5 min just prior to their preparation, their weight was measured accurately and ranged from 17.5 to 24.3 mg.

2.3. Sample preparation

All glass instruments were heated at 550 °C for 40 min and other materials such as plastics were carefully washed with chloroform–methanol. During all procedures of sample preparation, organic solvents of infinity pure grade and ultra-pure water were used to avoid contamination with trace fats and oils. The procedures were performed with slight modifications of methods previously reported [20]. Thus, washed hair samples were cut into small pieces with scissors, and were then immersed successively into 5 ml

chloroform–methanol 2:1, 1:1 and 1:2 (v/v), followed by chloroform–methanol–water 18:9:1 (v/v/v) each for 24 h at room temperature. The extracts were filtered through a 0.5 μm Millipore filter. The filtrates were united and dried under a nitrogen stream, and the residue (extractable lipids) was then dissolved in 100 μl chloroform–methanol 1:1 (v/v) for analysis by TLC/FID.

The delipidized pieces of hair fibers were saponified for 2 h at 60 °C by adding 5 ml 1 M KOH in 90% methanol. To the resulting solution, 2.5 ml water, 1.25 ml 6 M HCl and 20 ml chloroform were added and then mixed vigorously in a shaking apparatus for 30 min. After the solution was separated into two phases, the upper aqueous phase, which included the hair residues, was removed. The lower chloroform phase was washed with 4 ml water and then filtered through a 0.5 μm Millipore filter. The filtrate was dried under a nitrogen stream, and the residue (integral lipids) was then dissolved in 10 ml methanol for analysis by LC/MS.

2.4. Instruments

TLC/FID analysis was performed using an Iatroscan MK-5 analyzer (Iatron Laboratories, Tokyo, Japan), a silica gel-coated Chromarod SIII (Iatron Laboratories) and a microdispenser (Drummond Scientific Co., Broomall, PA, USA). Chromatographic data were processed with SIC 480 II software version 1.0 (System Instruments Corp., Tokyo, Japan). An Agilent 1100 Series LC/MSD SL (single quadrupole) system equipped with ChemStation software, a 1100-well plate autosampler (Agilent Technologies, Palo Alto, CA, USA) and a L-Column ODS 2.1 mm Ø × 150 mm (Chemical Evaluation and Research Institute, Tokyo, Japan) was used for LC/MS analysis. An Agilent 6890 GC Series coupled to an Agilent 5973 mass selective detector, which was equipped with ChemStation software (Agilent Technologies) and an Ultra Alloy #1 metal capillary column of 30 m × 0.25 mm Ø and a film thickness 0.15 μm with a guard column for 0.25 mm Ø (Frontier Lab, Fukushima, Japan), was used for analysis by GC/MS.

2.5. Chromatographic conditions

2.5.1. TLC/FID

The extractable lipids was dissolved in 100 μl chloroform-methanol 1:1 (v/v), and an appropriate volume (0.5–5 μl, usually 2 μl) of solutions containing the authentic lipids or extractable lipids was spotted onto the silica gel-coated rods using the microdispenser. The rods were developed consecutively three times using the following mobile phases: (i) hexane–diethyl ether–formic acid 50:20:2.5 (v/v/v) up to 6 cm; (ii) hexane–benzene 35:35 (v/v) up to 8 cm; (iii) hexane up to 10 cm. After each development, the rods were heated at 60 °C to dry the remaining solvent. After three developments and dryings were completed, the rods were processed through FID by a hydrogen flow of 160 ml/min, an air flow of 2.0 l/min, and a scanning speed of 30 s/scan. Calibration lines were produced with authentic lipids (squalene for SQ; RF = 0.68, palmityl palmitate for WEs; RF = 0.60, tripalmitin for TGs; RF = 0.48, and palmitic acid for FFAs; RF = 0.43).

2.5.2. LC/MS analysis

After the extractable lipid solution was used for TLC/FID analysis, the rest was dried under a nitrogen stream and dissolved again in 1000 μl methanol for the next LC/MS analysis. The injection volume was 5 μl of each sample solution (the extractable and integral lipids) and authentic lipid solution. The extractable lipids were analyzed for CS and the integral lipids only for 18-MEA. The column temperature was maintained at 40 °C and was eluted at a flow rate of 0.2 ml/min. The mobile phase for isocratic elution was methanol containing 100 mM ammonium acetate and 50 mM acetic acid. Electrospray ionization in the mass spectrometer was performed with the following settings: negative ion mode, V-cap voltage 4000 V, fragmenter voltage 200 V, a flow of heated dry nitrogen gas 11 l/min, and a heater temperature of 350 °C. The mass spectrometer was operated in selected ion monitoring (SIM) with m/z = 325 and 465 for 18-MEA or HEA and CS, respectively. Calibration lines were generated by plotting the concentration of authentic HEA and CS against their peak areas. In determining the level of 18-MEA, an alternative calibration line of HEA was used with the constant slope ratio (=1.05) of 18-MEA to that of HEA, as described in Section 3.

2.5.3. GC/MS analysis

After the extractable lipid solution was used for LC/MS analysis, the rest was dried under a nitrogen stream and dissolved again in 1000 μl chloroform–methanol 1:1 (v/v) for the next GC/MS analysis. Constant amounts of the internal standards BC (1.0 μg) and CER/MS (0.10 μg) were added to 100 μl of the extractable lipids, and then the solvent was evaporated. To the dried lipids, 150 μl of freshly dehydrated pyridine and 50 μl TMSI were added and trimethylsilylated (TMS) at 80 °C for 20 min. To the resulting solution, 100 μl water and 200 μl hexane were added and then mixed vigorously. The upper hexane phase was used as a sample solution and 1 μl was introduced into GC/MS via on-column injection. Helium was used as the carrier gas (0.8 ml/min). The oven temperature was programmed as follows: initial 50 °C for 1 min, from 50 to 200 °C at 50 °C/min, from 200 to 360 °C at 10 °C/min, and finally 360 °C for 5 min (25 min each run). The mass spectrometer was operated in electron impact mode at 70 eV. The temperatures of ion source and quadrupole were maintained at 150 and at 230 °C, respectively. The ions m/z = 443, 445, 313 and 342 were used in SIM for TMS-CH, TMS-BC, TMS-CER/PDS and TMS-CER/MS, respectively, with the time program as follows: SIM at 443 and 445 from 5 to 17 min, and at 313 and 342 from 17 to 20 min. Calibration lines were generated by plotting the concentration of authentic CH and CER/PDS against their peak ratios to the corresponding internal standards (BC and CER/MS).

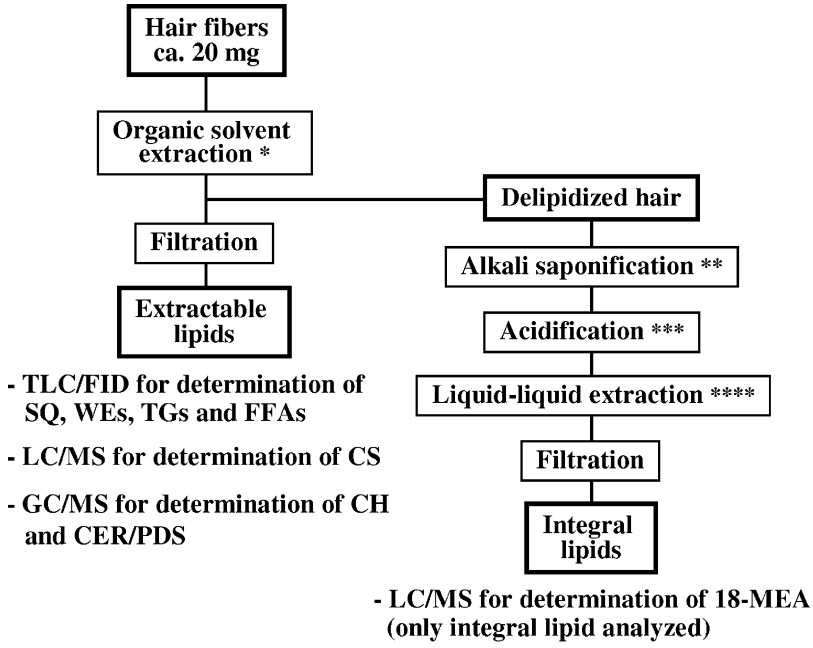

Fig. 1. Outline of analytical procedures for the determination of hair lipids by the systematic method developed in this study.

2.6. Characterization of hair lipid composition along the longitudinal direction

Each hair sample between the proximal root end and the distal tip was subjected to determination of hair lipids according to the procedures shown in Fig. 1. In the event that peaks of interest were out of the calibration line, the analyses were performed repeatedly with appropriate dilutions or concentrations of the sample solutions. All analyses were performed in duplicate and their average values are expressed as weight of lipids to that of hair fibers (mg/g hair).

3. Results

3.1. Optimization of TLC/FID conditions

Fig. 2 shows a representative TLC/FID chromatogram of extractable lipids from ca. 20 mg of hair fibers under optimized conditions. A broader peak of FFAs consisting of a mixture of various saturated and unsaturated alkyl chains was observed in Fig. 2. Since a similar peak of FFAs appeared in analysis of hair fibers with a lower level of FFAs, the broad peak of FFAs is not ascribed to overloading lipid applications. This may be attributed to a chromatographic behavior in which the degree of unsaturation in FFAs affects

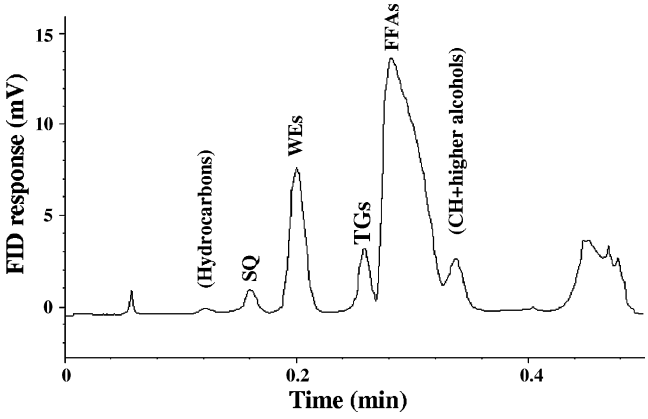

Fig. 2. A TLC/FID chromatogram of extractable lipids of hair fibers under optimal conditions for the determination of SQ, WEs, TGs and FAs. Sample: 100 μl chloroform–methanol (1:1 v/v) solution containing extractable lipids from ca. 20 mg of hair fibers that were obtained from a Japanese female volunteer. Experimental conditions: stationary phase, a silica gel-coated Chromarod SIII; applied volume, 2 μl; development: (i) hexane–diethyl ether–formic acid 50:20:2.5 (v/v/v) up to 6 cm; (ii) hexane–benzene 35:35 (v/v) up to 8 cm; (iii) hexane up to 10 cm; FID, hydrogen flow of 160 ml/min, air flow of 2.0 l/min and scanning speed of 30 s/scan.

their RF values in TLC using a non-polar mobile phase system [27]. Determination was performed for SQ, WEs, TGs and FFAs (except CH), which were approximately estimated as 0.8, 4.6, 1.4 and 11.2 mg/g hair, respectively. A multi-

Table 1
Reproducibility (R.S.D. in %, $n = 6$), limit of detections (LOD, S/N = 3), and calibration lines obtained for authentic lipids with TLC/FID, LC/MS and GC/MS

Lipid	Reproducibility (R.S.D.%)		LOD (ng)	Calibration line equation, R
	Retention time	Area (ratio)		
Squalene[a]	6.3	7.5	150	$y = 3.20x - 699$, 0.998
Palmityl palmitate[a]	3.9	6.0	50	$y = 7.58x - 1059$, 0.999
Tripalmitin[a]	2.4	3.8	50	$y = 8.56x - 1458$, 0.997
Palmitic acid[a]	2.3	6.5	50	$y = 8.51x - 941$, 0.997
CS[b]	0.02	1.3	0.002	$y = 10.57x - 0.09$, 0.999
18-MEA[b]	0.02	1.4	0.005	$y = 6.765x - 0.058$, 0.999
HEA[b]	0.02	1.5	0.005	$y = 6.437x - 0.068$, 0.999
CH[c]	0.01	0.6	0.03	$y = 0.593x - 0.242$, 0.999
CER/PDS[c]	0.01	5.4	0.01	$y = 0.537x - 0.009$, 0.998

[a] Analysis by TLC/FID. All calibration lines were produced with applications of 500, 1000, 1500, 2000 and 2500 ng of the authentics.
[b] Analysis by LC/MS. HEA is an alternative authentic lipid for the determination of 18-MEA. A calibration line for CS was produced with injections of 0.05, 0.25, 0.5, 0.75 and 1 ng of the authentic. Calibration line for 18-MEA and HEA were produced with injections of 0.05, 0.25, 0.5, 2.5 and 5 ng of the authentics.
[c] Analysis by GC/MS. Reproducibility of area ratios is shown. A calibration line for CH was produced with injections of 0.1, 0.5, 1, 5 and 10 ng of the authentic and with a constant amount (5 ng) of the internal standard BC. A calibration line for CER/PDS was produced with injections of 0.025, 0.05, 0.1, 0.5 and 1 ng of the authentic and with a constant amount (0.5 ng) of the internal standard CER/MS.

ple development system for the TLC rods was optimized to achieve mutual separation of SQ, WEs, TGs and FFAs, and other lipids. The first development with hexane–diethyl ether–formic acid 50:20:2.5 (v/v/v) to a distance of 6 cm provided separation between the non-polar (SQ, WEs, TGs and FFAs) and polar lipids (CH, CERs and CS). The second development with hexane–benzene 35:35 (v/v) up to 8 cm successfully separated WEs, TGs and FFAs. The third development with hexane up to 10 cm allowed the detection of only SQ by separating hydrocarbons included in the extractable lipids.

Table 1 presents validation data, including reproducibility, limit of detection (LOD) and calibration lines for authentic lipids, such as SQ, palmityl palmitate, tripalmitin and palmitic acid, under TLC/FID conditions optimized for the separation of SQ, WEs, TGs and FFAs in the extractable lipids. The R.S.D. for retention times and peak areas of six consecutive analyses of a 500 μg/ml authentic solution (application of 2 μl) was in the range of 2.3–6.3% and 3.8–7.5%, respectively. The LOD was 150 ng for squalene, and 50 ng for palmityl palmitate, tripalmitin and palmitic acid. All correlation coefficients were greater than 0.997 in all authentic lipids tested in the range of 500–2500 μg. The tentative accuracy was evaluated by adding known amounts of authentic lipids to the extractable lipids. After three replicates were obtained, the tentative recoveries at a time when the exemplary authentics were added into the extractable lipids were found to be: 102% for SQ, 93% for palmityl palmitate, 110% for tripalmitin and 94% for palmitic acid.

3.2. Optimization of LC/MS conditions

Our preliminary examination of LC conditions for CS and 18-MEA revealed that combining an ODS column and a mobile phase of methanol containing 100 mM ammonium acetate and 50 mM acetic acid provided good separation of CS and 18-MEA within 6 min. Thus, addition of 100 mM ammonium acetate into methanol was necessary to obtain a better shaped peak of CS, while the further addition of 50 mM acetic acid was effective in retaining a symmetric peak of 18-MEA (as well as HEA). Mass spectra of CS and 18-MEA were obtained in both the positive and negative ion mode with atmosphere pressure chemical ionization or electrospray ionization (ESI) under the mobile phase described above. Based upon these experiments, the negative ion mode of ESI was chosen due to the more abundant signals for both CS and 18-MEA. CS and 18-MEA provided the most abundant de-sodium molecular ion $[M - Na]^-$ and de-protonated one $[M - H]^-$, respectively (Fig. 3). Therefore, the molecular-related ions $m/z = 465$ for CS and $m/z = 325$ for 18-MEA were chosen as targets for SIM in this analysis. The ions, m/z 547 for CS and 407 for MEA were identified to be sodium acetate adduct ions, $[M + CH_3COONa - Na]^-$ and $[M + CH_3COONa - H]^-$, respectively. In this SIM condition for LC/MS, the parameters described in Section 2 were fixed for the optimal intensity of peak areas both for CS and for 18-MEA.

An alternative procedure for the convenient determination of 18-MEA was developed. According to its similarity to 18-MEA, heneicosanoic acid (HEA, ca. 99% by GC/FID) having 21 carbons and a straight alkyl chain, was used as a candidate. Intra- and inter-day variations for calibration lines of 18-MEA (96% purity) and HEA (99% purity), produced by plotting their concentrations in the range of 0.01–1 μg/ml against their peak areas with SIM of LC/MS, are shown in Table 2. All calibration lines had greater than 0.999 correlation coefficients, and the slope ratios of 18-MEA to HEA were constant (=1.05) while the intercept ratios were variable. Since the contribution of the intercept ratio to the absolute value is quite small, it can be ignored, and the level of 18-MEA (x) can be determined using the alternative authentic HEA, according to the following equation:

$$y = 1.05ax + b$$

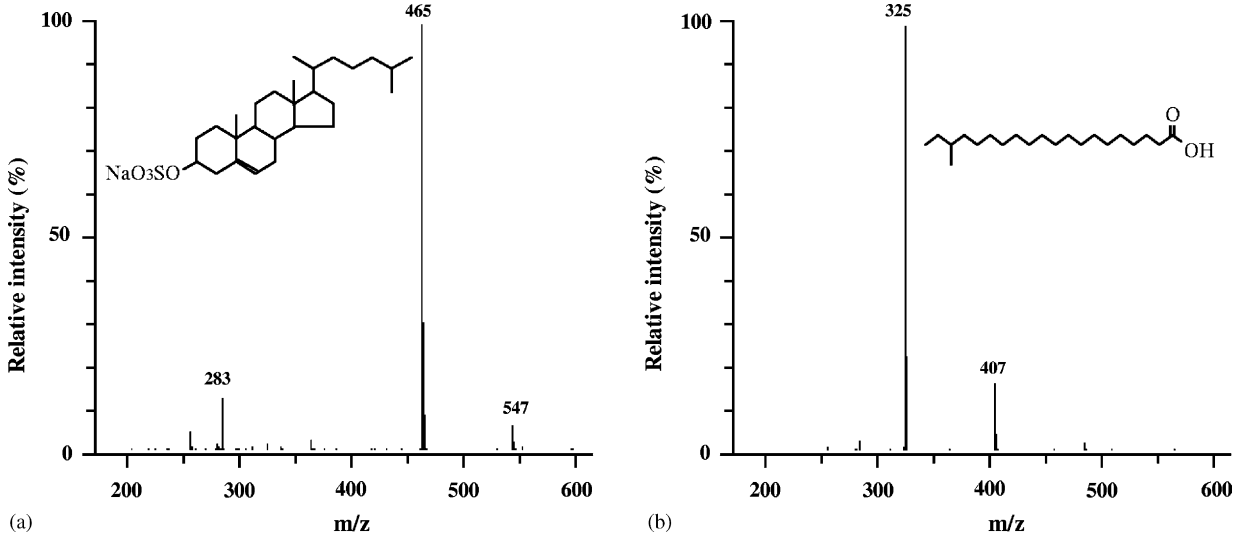

Fig. 3. Mass spectra of CS and 18-MEA with LC/MS with the negative ion mode of ESI. (a) CS and (b) 18-MEA. Sample: authentic CS and 18-MEA solutions at each concentration 0.5 μg/ml. Experimental conditions: column, L-Column ODS (2.1 mm Ø × 150 mm); column temperature, 40 °C; injection volume, 5 μl; mobile phase, methanol containing 100 mM ammonium acetate and 50 mM acetic acid at a flow rate of 0.2 ml/min; ionization, negative ESI; V-cap voltage, 4000 V; fragmenter voltage, 200 V; flow rate of heated dry nitrogen gas, 11 l/min; a heater temperature, 350 °C; scan range, m/z = 200–600.

where y is the peak area of interest of 18-MEA, a and b represent the slope and the intercept of HEA's calibration line produced prior to LC/MS analysis of 18-MEA in the integral lipids.

Fig. 4 shows representative LC/MS chromatograms of CS and 18-MEA detected in ca. 20 mg of hair fibers, and of authentic HEA under the optimal conditions with SIM, in which levels of CS and 18-MEA were 0.12 and 0.32 mg/g hair, respectively. The R.S.D. for retention times and peak areas of six consecutive injections of an authentic 0.1 μg/ml solution of CS and 0.5 μg/ml solution of 18-MEA and HEA, ranged within 0.02 and 1.5%, respectively, and the LOD were 0.002 ng for CS and 0.005 ng for 18-MEA and HEA (Table 1). The correlation coefficients of the calibration lines of 18-MEA and HEA were greater than 0.999 in the range of 0.05–5 ng while that of CS was greater than 0.999 in the range of 0.05–1 ng (Tables 1 and 2). Thus, up to a concentration of 0.2 μg/ml, the peak areas of CS measured were lower than those expected based on its regression line from 0.01 to 0.2 μg/ml. Therefore, the calibration line of CS had to be produced in the range of 0.01–0.2 μg/ml in subsequent quantitative analyses. When the accuracy was measured by the addition of known amounts of authentic lipids, the mean recovery was 101% for CS and 99% for 18-MEA (n = 3). All the sufficient validation data allow us to determine levels of CS and 18-MEA derived from hair fibers by this convenient (especially for 18-MEA), sensitive, precise and accurate LC/MS method.

3.3. Optimization of GC/MS conditions

Although there are several types of CERs in human hair, CER/PDS was chosen as a representative target for determination because it is included at the highest level in hair lipids [18]. In determining CH and CER/PDS by GC/MS, the internal standards BC (β-cholestanol) and CER/MS (*N*-myristoyl sphingosine) were also used due to their analogous molecular structures. Under GC/MS conditions optimized as

Table 2
Inter- and intra-day variation for calibration lines of 18-MEA and HEA in the range of concentrations 0.01–1 μg/mL in SIM of LC/MS

	Equation, R		Ratio of 18-MEA-to-HEA	
	18-MEA	HEA	Slope	Intercept
Day 1				
n = 1	$y = 6.765x - 0.058$, 0.999	$y = 6.437x - 0.068$, 0.999	1.051	0.853
n = 2	$y = 6.697x + 0.057$, 0.999	$y = 6.402x + 0.062$, 0.999	1.046	0.919
Day 2				
n = 1	$y = 6.588x + 0.055$, 1.000	$y = 6.245x + 0.078$, 0.999	1.055	0.705
n = 2	$y = 6.514x + 0.062$, 0.999	$y = 6.169x + 0.072$, 0.999	1.056	0.861
Day 3				
n = 1	$y = 6.506x + 0.063$, 0.999	$y = 6.173x + 0.071$, 0.999	1.054	0.887
n = 2	$y = 6.346x + 0.062$, 0.999	$y = 6.032x + 0.079$, 0.999	1.052	0.785

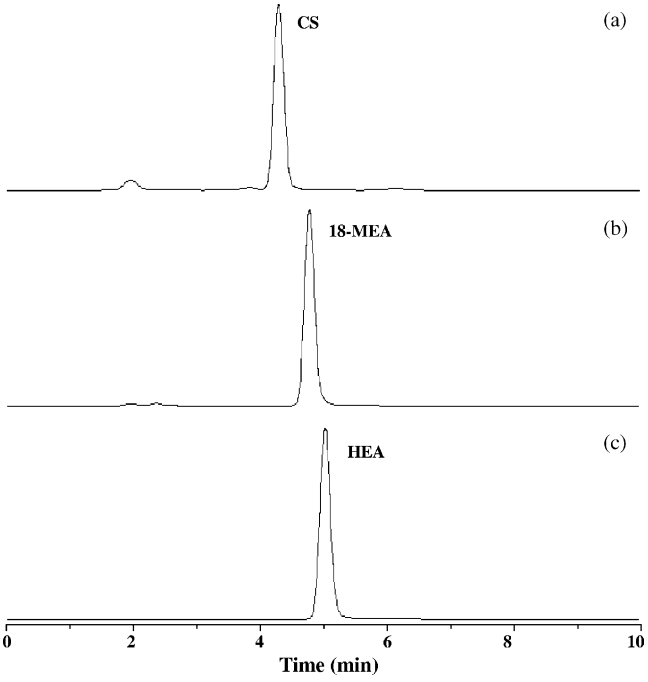

Fig. 4. LC/MS chromatograms of CS and 18-MEA in extractable lipids of hair fibers and an authentic HEA with the negative ion mode of ESI under optimal conditions. (a) SIM chromatogram at $m/z = 465$ for CS. (b) SIM chromatogram at $m/z = 325$ for 18-MEA. (c) SIM chromatogram at $m/z = 325$ for HEA. Sample: 1000 μl methanol solution containing extractable lipids from ca. 20 mg of hair fibers that were obtained from a Japanese female volunteer in (a) and (b), and authentic HEA solution at a concentration 0.5 μg/ml. Experimental conditions: see Fig. 3 except for SIM with $m/z = 465$ for CS and 325 for 18-MEA and HEA.

described later, no peak of natural CER/MS in hair fibers was detected.

Mass spectra for TMS-CH, TMS-BC, TMS-CER/PDS and TMS-CER/MS are shown in Fig. 5. TMS-CH provided the ions $m/z = 458$, 443, 368, 353 and 329, where $[M]^+$ (molecular ion) for 458, $[M-15]^+$ (loss of CH_3 from a TMS group) for 443, $[M-90]^+$ (loss of a trimethylsilanol) for 368, $[M-105]^+$ (loss of both a trimethylsilanol and CH_3) for 353 and $[M-129]^+$ (loss of $(CH_3)_3SiO{=}CH{-}CH{=}CH_2$) for 329 [28] were identified (Fig. 5(a)). As for the corresponding internal standard TMS-BC, the ions $m/z = 460$, 445, 403, 370 and 355 were observed, where $[M]^+$ for 460, $[M-15]^+$ for 445, $[M-57]^+$ (cleavage between C_{23} (charge retention) and C_{24}) for 403, $[M-90]^+$ for 370 and $[M-105]^+$ for 355 were identified (Fig. 5(b)). On the other hand, TMS-CER/PDS provided the ions $m/z = 668$, 580, 443, 370 and 313, where $[M-15]^+$ (loss of CH_3 from a TMS group) for 668, $[M-103]^+$ (loss of CH_2OTMS with cleavage between C_1 and C_2 of the dihydrosphingosine base) for 580, $[M-240]^+$ (cleavage between C_2 (charge retention) and C_3 with transfer of a TMS group) for 443 [29], $[M-313]^+$ (cleavage between C_2 (charge retention) and C_3) for 370 and $[M-370]^+$ (cleavage between C_2 and C_3 (charge retention)) for 313 were identified (Fig. 5(c)). In TMS-CER/MS, the characteristic ions $m/z = 638$, 426, 342 and 311 were recognized, where $[M-15]^+$ for 638, $[M-227]^+$ (loss of acylamide) for 426 [29], $[M-311]^+$ for 342 and $[M-342]^+$ for 311 were identified (Fig. 5(d)). The SIM ions, $m/z = 443$ for TMS-CH, 445 for TMS-BC, 313 for TMS-CER/PDS and 342 for TMS-CER/MS, were chosen in order to practice simultaneous determination of CH and CER/PDS in the extractable hair lipids with GC/MS. Thus, because the level of CH in the extractable lipids is always quite higher than that of CER/PDS, the less abundant ion ($m/z = 443$) for TMS-CH and the corresponding ion ($m/z = 445$) for TMS-BC were used. In contrast, the most abundant ion, $m/z = 313$, was used for TMS-CER/PDS. Although the corresponding ion m/z = 311 for TMS-CER/MS was used at first, the ion $m/z = 342$ was eventually chosen due to its lack of interference with other peaks existing in the extractable lipids.

Under the conditions optimized for SIM analysis of GC/MS, splitless injection of 1 μl at an injection temperature 370 °C was tested for the determination of CH and CER/PDS with the internal standards. However, this approach did not yield an acceptable sensitivity and precision for determining of CER/PDS (the LOD was only 0.2 ng CER/PDS at S/N = 3, and the R.S.D.% of the area ratio was 15.8% in consecutive injections of 0.5 μg/ml solution), which is probably attributable to the thermal-decomposition of TMS-CER/PDS due to the high temperature of the inlet. Therefore, a new approach with on-column injection, which can be expected to cause no thermal-decomposition owing to the inlet at a lower temperature, was examined by connecting a guard column of 0.25 mm Ø to the capillary column. This approach was a significant improvement over the previous one with splitless injection, showing elevated sensitivity and precision for the analysis of CER/PDS. Fig. 6 shows representative GC/MS chromatograms of TMS-CH and TMS-CER/PDS from ca. 20 mg of hair fibers, and their internal standards (TMS-BC and TMS-CER/MS) with SIM under optimal conditions, with CH and CER/PDS at 0.56 and 0.098 mg/g hair, respectively. Although a large peak of +2 isotope of TMS-CH in Fig. 6(b) was eluted just before TMS-BC, and there were several peaks that seem to be isomers of C18-dihydrosphingosine-based CERs in Fig. 6(c), these peaks did not interfere with our peaks of interest.

The R.S.D. for the retention times and the peak area ratios, with six consecutive injections of an authentic 5 μg/ml solution of CH and a 0.5 μg/ml solution of CER/PDS, ranged within 0.01% and from 0.6 to 5.4% (Table 1). The LOD were 0.03 ng for CH and 0.01 ng for CER/PDS, and each calibration line using the internal standard showed correlation coefficients of 0.999 for CH in the range of 0.1–10 ng and 0.998 for CER/PDS in the range of 0.025–1 ng (Table 1). All these validation data for CH and CER/PDS were sufficient for their determination, although the R.S.D. for the peak area ratios of CER/PDS and the linearities of the calibration lines were relatively lower. When the accuracy was examined by spiking known amounts of authentic lipids, the mean recovery was 105% for CH and 110% for CER/PDS ($n = 3$), which were also sufficient. By this optimization, a sensitive, precise

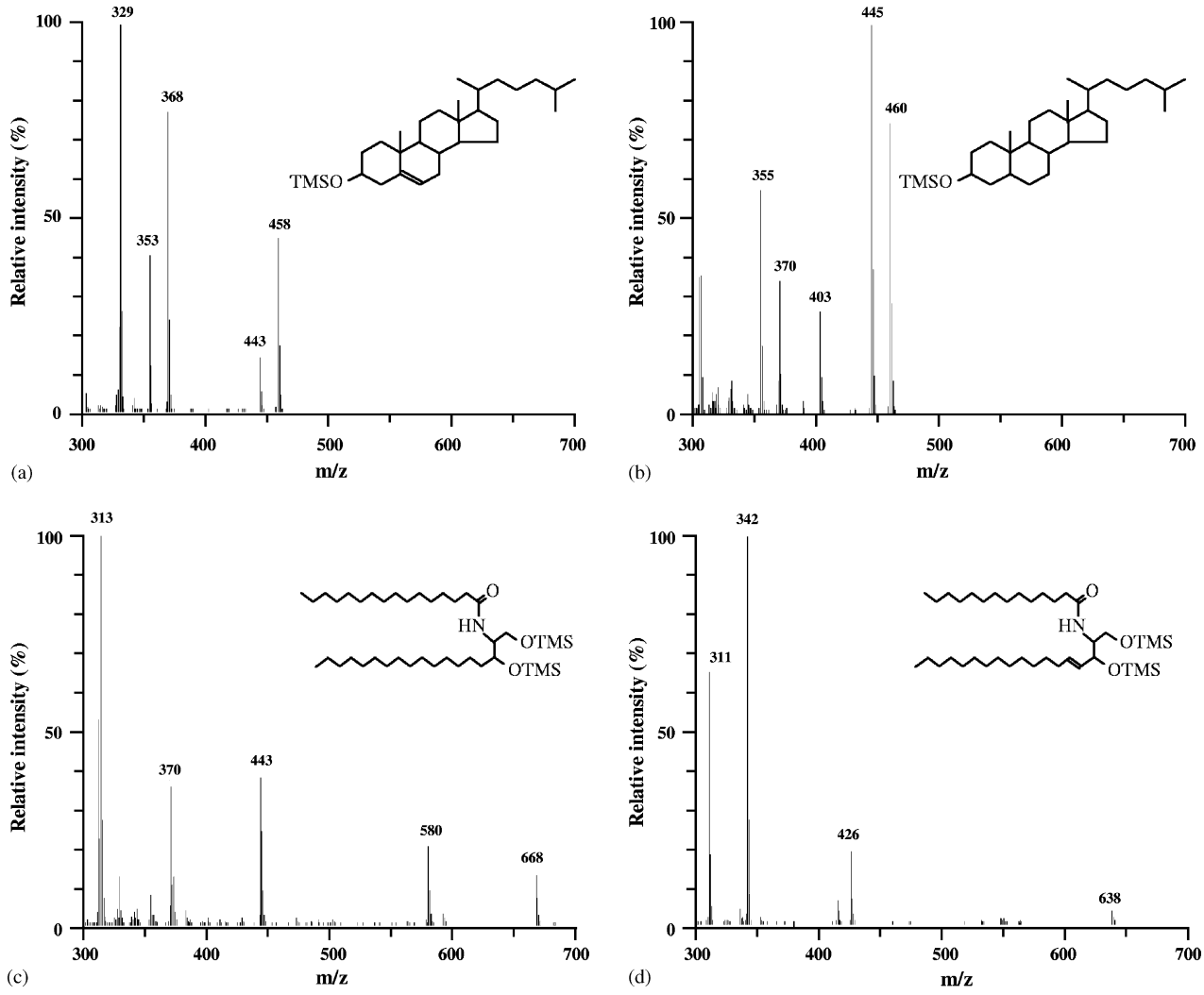

Fig. 5. Mass spectra of TMS-CH (a), TMS-BC (b), TMS-CER/PDS (c) and TMS-CER/MS (d) in GC/MS analysis. Sample: authentic CH, BC, CER/PDS and CER/MS at each concentration 5 μg/ml, which were trimethylsilylated. Experimental conditions: column, Ultra Alloy #1 metal capillary column (30 m × 0.25 mm Ø and a film thickness 0.15 μm) with guard column for 0.25 mm Ø; column temperature program, see Section 2; injection volume, 1 μl via on-column; carrier gas, helium at flow rate of 0.8 ml/min; ionization, electron impact mode at 70 eV; ion sorce temperature, 150 °C; quadrupole temperature, 230 °C; scan range, *m*/*z* = 300–700.

and accurate GC/MS method for the simultaneous determination of CH and CER/PDS derived from hair fibers has been established.

3.4. Application of the present method to characterize hair lipids

The systematic method for the determination of hair lipids, developed in this study was applied to analyze all hair lipids from the proximal root end to the distal tip of chemically untreated hair fibers (Hair-A and Hair-B). Hair-A and Hair-B were approximately 22 and 39 cm in length, respectively. Hair-A was cut into six pieces of 0–4 cm, 4–8 cm, 8–12 cm, 12–16 cm, 16–20 cm and more than 20 cm from the root end while Hair-B was cut into 10 pieces of 0–4 cm, 4–8 cm, 8–12 cm, 12–16 cm, 16–20 cm, 20–24 cm, 24–28 cm, 28–32 cm, 32–36 cm and more than 36 cm. These samples ranged from 17.5 to 24.3 mg in weight per piece.

Table 3 shows lipid levels of the six pieces of Hair-A and the 10 pieces of Hair-B. Although the level of SQ in Hair-A was obscure because it was below the LOD, the levels of other lipids along the distance of the hair from the root end were distinct. Thus, the middle regions of the hair fibers had the highest levels of SQ in Hair-B, WEs in Hair-A and Hair-B, and TGs and FFAs in Hair-A in all regions. The levels of TGs in Hair-B increased along the longitudinal direction while the levels of FFAs in Hair-B decreased. The levels of intrinsic endogenous 18-MEA, CS, CH and CER/PDS in Hair-A and in Hair-B consistently decreased with the distance along the hair from the root end without exception (Fig. 7), although there were various patterns of decreases. For 18-MEA, the gradual decreasing pattern in Hair-A was similar to that in Hair-B (Fig. 7(a)). The CS level

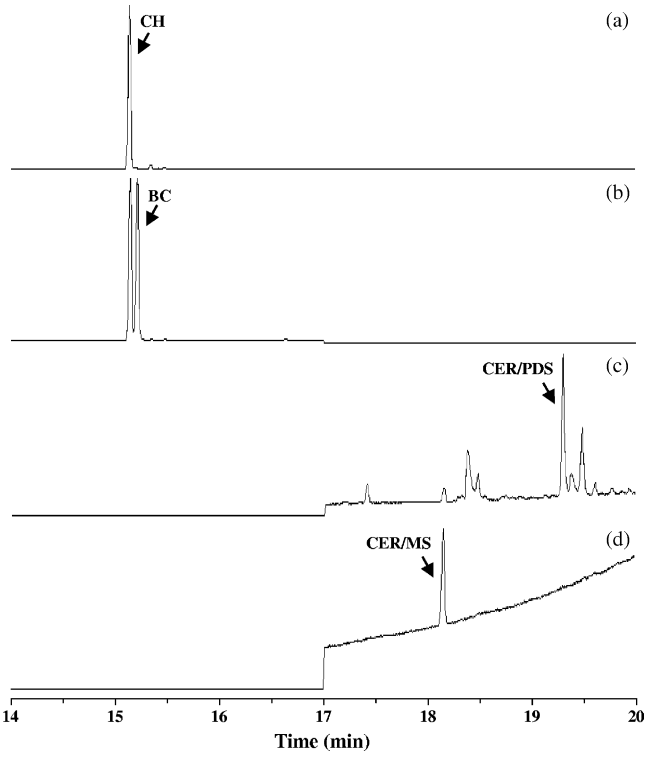

Fig. 6. GC/MS chromatograms of CH and CER/PDS in extractable lipids of hair fibers and their corresponding internal standards (BC and CER/MS) under optimal conditions. (a) SIM chromatogram at $m/z = 443$ for CH. (b) SIM chromatogram at $m/z = 445$ for the internal standard BC. (c) SIM chromatogram at $m/z = 313$ for CER/PDS. (d) SIM chromatogram at $m/z = 342$ for the internal standard CER/MS. Sample: 200 μl hexane solution containing extractable lipids from ca. 20 mg of hair fibers that were obtained from a Japanese female volunteer and added internal standards (BC and CER/MS), which were trimethylsilylated. Experimental conditions: see Fig. 5 except for SIM with $m/z = 443$ for TMS-CH and 445 for TMS-BC (data acquisition of 5–17 min) and 313 for TMS-CER/PDS and 342 for TMS-CER/MS (17–20 min).

in the hair near 20 cm decreased by ∼50% and by less than 30% of the level at the root end in Hair-A and in Hair-B, respectively (Fig. 7(b)). For the level of CH, the originally higher levels of CH in Hair-A decreased drastically near the root region while the originally lower levels of CH in Hair-B decreased gradually from the root to the tip (Fig. 7(c)). The level of CER/PDS showed contrasting patterns of CH between Hair-A and Hair-B. Thus, the level of CER/PDS was originally higher in Hair-B than in Hair-A, and the higher level of CER/PDS in Hair-B decreased drastically along the longitudinal direction compared with Hair-A (Fig. 7(d)).

4. Discussion

The objective of this study was to develop a systematic method for the sensitive, precise and accurate determination of all lipids including the trace levels of intrinsic endogenous CS, 18-MEA, CH and CER/PDS, using small amounts of hair fibers. TLC/FID conditions for the determination of SQ, WEs, TGs and FFAs were optimized by referring to the analytical conditions previously reported for analysis in wool [30–32]. The analytical conditions were also validated using palmityl palmitate for WEs, tripalmitin for TGs and palmitic acid for FFAs. However, in this TLC/FID, it should be noted that these validation data are tentative because WEs, TGs and FFAs are all complex mixtures consisting of various saturated and unsaturated species, each of which has a different response factor (e.g., relative response factors for stearic acid and oleic acid are 0.94 and 0.73, respectively, where that for palmitic acid is 1.00 (our unpublished data)). This means that levels of WEs, TGs and FFAs by this TLC/FID are approximate in hair fibers. For the determination of CS and 18-MEA by LC/MS, the optimization of both LC conditions

Table 3
Lipid levels of chemically untreated hair fibers from the proximal root end to the distal tip end

Distance sample from the root end (cm)	Hair (mg/g)							
	SQ	WEs	TGs	FFAs	18-MEA	CS	CH	CER/PDS
Hair-A								
0–4	<0.2	0.8	0.2	9.6	0.34	0.43	1.15	0.09
4–8	<0.2	2.8	0.5	15	0.36	0.34	0.41	0.09
8–12	<0.2	3.7	0.5	13	0.34	0.37	0.35	0.08
12–16	<0.2	4.3	0.3	12	0.32	0.32	0.33	0.08
16–20	<0.2	3.7	0.3	9.0	0.30	0.21	0.34	0.05
20	<0.2	3.5	0.4	9.1	0.28	0.23	0.33	0.06
Hair-B								
0–4	<0.2	4.1	0.2	23	0.35	0.36	0.50	0.17
4–8	<0.2	9.2	0.4	22	0.32	0.18	0.35	0.12
8–12	0.3	9.0	0.7	13	0.31	0.13	0.29	0.09
12–16	0.6	10	1.2	14	0.29	0.10	0.26	0.06
16–20	0.3	11	1.0	10	0.27	0.11	0.25	0.07
20–24	0.4	8.9	0.9	7.9	0.26	0.11	0.25	0.06
24–28	0.5	8.5	1.4	7.4	0.23	0.09	0.24	0.05
28–32	0.2	8.8	1.5	8.2	0.20	0.05	0.25	0.05
32–36	<0.2	6.4	1.4	6.6	0.18	0.05	0.27	0.08
36	<0.2	5.4	1.4	5.7	0.12	0.05	0.29	0.07

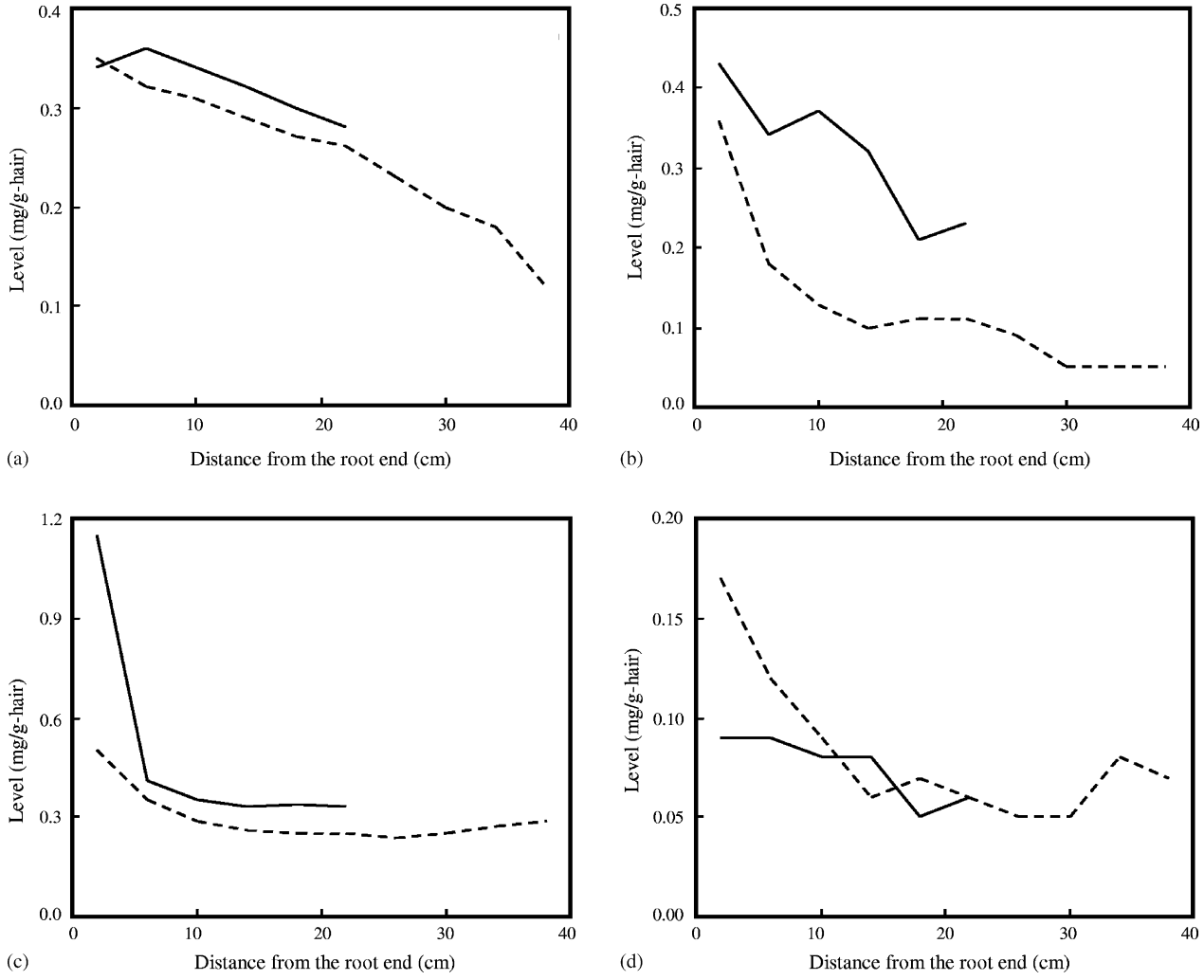

Fig. 7. Relationships between the distance from the proximal root end and the levels of intrinsic endogenous lipids such as 18-MEA (a), CS (b), CH (c) and CER/PDS (d). Straight lines and broken lines show data in Hair-A and Hair-B, respectively.

and MS conditions have provided us with satisfactory LOD, reproducibility, recovery and calibration lines. Although the dynamic range for CS was narrower than that for 18-MEA, it does not hinder practical measurements. In addition, an alternative procedure for the determination of 18-MEA using a calibration line of HEA has been established. This alternative procedure under the analytical conditions of LC/MS allows a convenient use of commercially available HEA as an authentic calibration standard without 18-MEA that is not commercially available, although 18-MEA can be also determined using a calibration line of the authentic 18-MEA like the other lipids. For GC/MS with suitable LOD, reproducibility, recovery and calibration lines, the optimal selection of SIM ions for TMS-CH and TMS-CER/PDS enabled their simultaneous determination in a single run, and the adoption of on-column injection improved the reproducibility of TMS-CER/PDS that has a higher boiling point in GC. However, in this GC/MS, it should be noted that a level of CER/PDS obtained represents only a criterion for CERs in hair fibers. Therefore, a systematic method for the sensitive and specific determination of all hair lipids in combination with TLC/FID, LC/MS and GC/MS, using hair fibers as small as 20 mg, has been established, although there are some quantitative limitations of TLC/FID for WEs, TGs and FFAs, and of GC/MS for CER/PDS.

To our knowledge, this is the first time that LC/MS has been used to detect and determine hair lipids. Furthermore, there have been no reports on the sensitive determination of trace hair lipids with SIM of GC/MS although it has been used to analyze their molecular structures based upon scan measurements [13,17,18]. As for the determination of 18-MEA, GC/FID [13,17] with methylation and LC/FLD [20,21] with fluorescent derivatization have been used, while the level of CS has been determined by GC/FID accompanied by extremely complicated pre-treatments [16]. However, our LC/MS method can simultaneously determine 18-MEA and CS without complicated pre-treatments. On the other hand, the relatively simple GC/FID methods, with or without trimethylsilylation, for the determination of CH in hair lipids have been reported [6,16,20], and very troublesome and

time-consuming methods to determine CERs in hair fibers have been developed that are based upon GC/FID [18] and LC/FLD [20]. However, we have determined both CH and CER/PDS in hair fibers very easily by our GC/MS method. Here, we have established a systematic method in combination with TLC/FID, LC/MS and GC/MS, as summarized in Fig. 1. This established method is superior to a conventional systematic method previously reported [20]. Thus, firstly, CS is included as hair lipids in this method, while it is not included in the conventional method. Secondly, this established method is simpler and easier in analytical procedures than the conventional method that is troublesome and time-consuming. Thirdly, in this established method, the amounts of hair fibers necessary for analyses of all hair lipids can be reduced to 20 mg compared with approximately 100 mg required for conventional methods. The usefulness of these methods was evidenced by its application to analysis of all hair lipids from the root end to the tip in chemically untreated hair fibers (Hair-A and Hair-B).

The levels of SQ, WEs, TGs and FFAs in Hair-A or in Hair-B show various changes from the root end to the tip, while intrinsic endogenous lipids such as CS, 18-MEA, CH and CER/PDS, consistently decrease along the distance from the root end. The levels of SQ, WEs, TGs, FFAs, CH and 18-MEA at the proximal root (0–4 cm) are not contradictory to our previous data of the proximal root regions in 44 Japanese females, which are <0.2–3.5 mg/g hair for SQ, 0.3–17 for WEs, <0.2–4.6 for TGs, 2.4–56.2 for FFAs, 0.4–2.8 for CH and 0.22–0.47 for 18-MEA [20]. However, it is likely that there are no studies that have characterized the levels of hair lipids from the root end to the tip, except for work by Kon et al. [33] who determined only the levels of 18-MEA in chemically untreated hair fibers. On the other hand, changes in the amino acid composition and in the morphology of hair fibers along the longitudinal direction, which may be due to external factors such as shampooing, light, heat, brushing and/or wetting/drying, are well known [34–37]. Thus, in the amino acid composition, cystine decreases while cysteic acid increases with the longitudinal distance [34]. Along their distance from the root end, morphologically, a smooth cuticle edge becomes jagged [35], followed by a decrease in cuticle cell layers [36], while voids or holes within hair fibers increase [37]. Therefore, some cases in which the levels of exogenous SQ, WEs, TGs and FFAs increase based upon the distance from the root end might be caused by the penetration of sebum following the breakage of structures in hair fibers as hair grows, because these lipids originate from excreted sebum [12,17,20]. On the other hand, it has been speculated that endogenous CH, CER/PDS, 18-MEA and CS (which are originally biosynthesized in hair matrix cells) are gradually lost, accompanied by the breakage of cross-linking structures and morphology in hair fibers from the root end to the tip. In particular, a decrease of 18-MEA chemically bound to the cuticle surface by a thioester linkage [21] may accurately reflect morphological changes of the cuticle [35,36]. Thus, it seems reasonable to assume that changes in the hair lipid composition in longitudinal regions along the distance from the root end may correspond to their origins. Therefore, the present method has proven to be useful to clarify the behaviors of exogenous and endogenous hair lipids on the scalp. Meanwhile, from the viewpoint of individual differences, distinct behaviors at the level of intrinsic endogenous lipids (except for 18-MEA) were observed, as shown in Fig. 7, in spite of their consistent decreases along the longitudinal direction. This suggests that the present method would be useful to characterize the hair lipid composition of individuals whose hair has been exposed to various external stresses. Although only Hair-A and Hair-B collected from two different females were analyzed in this study, it would be expected that in the future we could approach some speculations or understandings about the functions of the CMC since the lipid composition of hair fibers from various individuals might be related to their physicochemical properties such as the diffusion of dyestuffs, the water content at various humidity and the deletion of cuticle layers.

In conclusion, a systematic method has been developed for the determination of all hair lipids, including trace intrinsic endogenous ones, in combination with TLC/FID, LC/MS and GC/MS. This method is applicable to determination of all lipids in amounts as small as 20 mg of hair fibers, which is superior to conventional methods that require 100 mg or more. As shown in our study, the lipid composition along the distance from the proximal root end of the hair fibers has been characterized for the first time, and demonstrates that specific changes for each hair lipid depend upon the origins of hair lipids and upon individuals whose hairs have been exposed to various external stresses. This method should contribute to future research on characterizing relationships between the levels of intrinsic endogenous lipids and the physicochemical properties of diverse hair fibers exposed to various external factors. This should lead in turn to clarifying the important roles of intrinsic endogenous 18-MEA, CS, CH and CERs in the function of the CMC.

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