methods

Liquid chromatography-mass spectrometry for comprehensive profiling of ceramide molecules in human hair

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Abstract **Ceramides (CERs) play key roles in signal transduction and cell regulation, probably during the keratinization of human hair. Current methods using mass spectrometry (MS), however, are not sufficient to allow the comprehensive analysis of CER molecules, including isobaric and isomeric CERs. Therefore, a method for the comprehensive profiling of CERs was developed. The method developed is based on reversed-phase liquid chromatography (RPLC) coupled to atmospheric pressure chemical ionization (APCI)-MS. Comprehensive identification and profiling of CERs is achieved using two sets of multimass chromatograms obtained from two channel detections that monitor both molecular-related and sphingoid-related ions under two different in-source collision-induced dissociation conditions and using retention times obtained from RPLC. The application of this method revealed that human hair contains 73 species of CER molecules, which were all corroborated by structural analysis using tandem mass spectrometry. The results further revealed that the composition is characterized by predominant molecules consisting of even carbon atom-containing saturated/unsaturated nonhydroxy or α-hydroxy fatty acids and C_{18} dihydrosphingosine, a minor but distinct content of isobaric/isomeric and odd chain-containing CERs. This successfully developed RPLC-APCI-MS technique allows the comprehensive profiling of CER molecules in hair for the investigation of their physicochemical and physiological roles.**—Masukawa, Y., H. Tsujimura, and H. Narita. **Liquid chromatography-mass spectrometry for comprehensive profiling of ceramide molecules in human hair. *J. Lipid Res.* 2006.** 47: **1559–1571.**

Supplementary key words fatty acid moiety • identification • isobaric • isomeric • molecular-related ion • multimass chromatogram • retention time • sphingoid moiety • sphingoid-related ion

Ceramides (CERs), which consist of a long amino alcoholic chain [sphingoid moiety (SPM)] covalently bound via an amide linkage to a fatty acid moiety (FAM), have important physicochemical roles in the barrier function and water-holding property of skin, together with other lipids in intercellular spaces of the stratum corneum (1, 2). They also play key roles in signal transduction and cell regulation relevant to cell growth arrest, differentiation, senescence, apoptosis, and immune responses (3–9). Human cells/tissues predominantly include CERs consisting of an even carbon atom-containing nonhydroxy FAM and a C_{18} sphingosine moiety together with less abundant CERs consisting of an even carbon atom-containing α-hydroxy FAM and a C_{18} sphingosine moiety (10–15). Among these CERs, it is thought that the molecules with C_{16} or C_{24} nonhydroxy FAMs may be relevant to apoptosis (16–18). The possibility that specific CER molecules may be involved with human diseases such as sphingolipidosis and peroxisomal disorders has also been proposed (19, 20). Although only predominant CERs are currently targeted, less abundant but bioactive CERs should also be comprehensively studied.

There are extremely complex CERs consisting of nonhydroxy, α-hydroxy, or ω-hydroxy FAMs and SPMs, such as sphingosine, dihydrosphingosine, phytosphingosine, or 6-hydroxy-sphingosine, in human stratum corneum (21). Some dermatologists are interested in the dynamics of CER-related lipids in differentiated keratinocytes where apoptosis occurs in skin (22). On the other hand, human hair also includes CERs that are predominantly composed of a nonhydroxy or α-hydroxy FAM and a dihydrosphingosine moiety (23, 24). We hypothesize that the CERs may be related to apoptosis during keratinization that proceeds from living hair matrix cells to dead cuticular or cortical

Abbreviations: ADS, ceramides consisting of α-hydroxy fatty acid and dihydrosphingosine moieties; APCI, atmospheric pressure chemical ionization; AS, ceramides consisting of α-hydroxy fatty acid and sphingosine moieties; CER, ceramide; CID, collision-induced dissociation; ESI, electrospray ionization; FAM, fatty acid moiety; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; NDS, ceramides consisting of nonhydroxy fatty acid and dihydrosphingosine moieties; NS, ceramides consisting of nonhydroxy fatty acid and sphingosine moieties; RPLC, reversed-phase liquid chromatography; RT, retention time; SPM, sphingoid moiety; TIC, total ion chromatogram.

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cells, ascribed to their bioactivities, and may contribute to barrier function and water holding in hair. To drive such a study, we need an analytical method for the comprehensive profiling of all CER molecules in hair, including not only the predominant CERs but also isobaric CERs and less abundant α-hydroxy FAM-containing CERs.

Many methods, such as thin-layer chromatography (25), diacylglycerol kinase assay (26), liquid chromatography (LC) connected to evaporative light-scattering detection (27), and LC connected to ultraviolet light detection using a derivatizing technique (28), have been used to analyze CERs. However, these conventional methods are not sufficient to comprehensively detect CER molecules in biological materials, despite being useful for the determination of total, type, and/or molecular levels of interesting CERs. Although gas chromatography-mass spectrometry (MS) can individually detect trimethylsilylated CERs (23, 24), it cannot comprehensively detect minor CERs as well as major CERs because of its lower sensitivity. Since Mano et al. (29), Gu et al. (30), and Couch et al. (31) demonstrated MS using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) to be extremely valuable tools in the analysis of mixed CER molecules, many methods for the identification and determination of CER molecules have been proposed and applied to the analysis of CERs in human cells/tissues (10, 32–45): reversed-phase liquid chromatography (RPLC) (34, 35, 39, 40), normal-phase LC (41–43, 45) connected to single quadrupole MS (39, 40, 44) or ion trap MS (34, 35, 41–43, 45), and direct triple quadrupole MS (10, 32, 33, 36–38). However, these methods do not provide comprehensive profiling of all CER molecules, including isobaric and isomeric CERs. Furthermore, it is unknown whether those methods are optimal to detect lower levels of α-hydroxy FAM-containing CERs that are present in human cells/tissues, because those CERs were not detected in any of their applications (10, 32–45), despite their successful detection in authentic samples.

The aim of this study was to develop a method for the comprehensive profiling of CER molecules using LC-MS. Consequently, this method was successfully developed by optimizing RPLC connected to APCI-MS in the positive ion mode, which uses two channel detections that monitor both molecular-related and SPM-related ions under two different in-source collision-induced dissociation (CID) conditions. Here, we show that the composition of CERs in hair is characterized not only by predominant CER molecules consisting of saturated/unsaturated FAMs with even numbers of carbon atoms and a C_{18} dihydrosphingosine moiety but also by isobaric or isomeric CERs, α-hydroxy fatty acid-containing CERs, and odd chain-containing CERs.

MATERIALS AND METHODS

Nomenclature

CERs are termed according to Motta et al. (46). They consist of a nonhydroxy FAM and a dihydrosphingosine moiety (NDS), a nonhydroxy FAM and a sphingosine moiety (NS), an α-hydroxy FAM and a dihydrosphingosine moiety (ADS), or a α-hydroxy FAM and a sphingosine moiety (AS). Based on this terminology, each CER molecule is individually termed as follows: the number of FAM carbon atoms and unsaturation (if present) is expressed after the letter N or A, whereas the number of SPM carbon atoms is expressed after the letter(s) DS or S (e.g., N24:1DS18 for a CER molecule with nervonic acid and C_{18} dihydrosphingosine moieties, N18DS20 for stearic acid and C_{20} dihydrosphingosine, and A16DS18 for α-hydroxypalmitic acid and C_{18} dihydrosphingosine).

Chemicals

Methanol and chloroform, of infinity pure grade from Wako Pure Chemical Industries (Tokyo, Japan), and *n*-hexane, of HPLC grade from Kanto Reagents (Tokyo, Japan), were used to prepare the hair lipid samples. Methanol and 2-propanol, of HPLC grade from Kanto Reagents, were used to prepare the mobile phase solution in RPLC-APCI-MS. Ultrapure water prepared using the Milli-Q purification system (Millipore, Bedford, MA) was used in all procedures. Authentic CERs, such as N-16:0 sphinganine (N16DS18), N-24:0 sphinganine (N24DS18), CER C16:0 (N16S18), and CER C24:0 (N24S18), were from Avanti Polar Lipids (Alabaster, AL). Stock individual authentic solutions (1 mg/ml) were prepared by dissolving accurate amounts of each authentic CER in chloroform-methanol (1:9, v/v) and were stored at −4°C. Working authentic lipid mixtures were obtained by further dilution of stock solutions with chloroform-methanol (1:9, v/v) just before use.

Hair fibers and preparations

The protocol was approved by the Ethical Committee of the Kao Corporation of Japan, based on the recommendations from the Declaration of Helsinki. Written informed consent was obtained from all volunteers. Scalp hair fibers were collected from five Japanese volunteers (designated Hair-A for a female aged 15 years, Hair-B for a female aged 20 years, Hair-C for a female aged 33 years, Hair-D for a female aged 51 years, and Hair-E for a male aged 40 years). Hair-E was collected as two separate groups: black and gray fibers (Hair-Eb and Hair-Eg). None of these hair samples had ever been treated with chemical reactions such as coloring and permanent waving during daily life activities. Only the 5 cm of fibers distant from the proximal root ends (∼100 mg for Hair-A and ∼50 mg for the others) was obtained. The fibers were washed with plain shampoo for 1 min and rinsed with water for 10 min, which was repeated twice. The dried fibers were washed with *n*-hexane for 5 min to remove lipids contaminating the surface, and their weight was measured accurately after drying at room temperature for 24 h. The *n*-hexane washing for 5 min was determined based on our previous experiment, which suggested that little internal lipids in hair are removed by *n*-hexane washing for <5 min (47). The preparative procedures were performed according to our previous reports (24, 47). Thus, the washed fibers were immersed successively into 5 ml of 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 combined and dried under a nitrogen stream, and the residue was then dissolved in 0.5 ml of chloroform-methanol (1:9, v/v).

MS analysis

An Agilent 1100 series LC/MSD SL (single quadrupole) system equipped with an ESI or APCI source, ChemStation software, an 1100 well plate autosampler (Agilent Technologies, Palo Alto,

CA), and a L-column ODS column (2.1 mm × 150 mm; Chemical Evaluation and Research Institute, Tokyo, Japan) were used. For the flow injection MS analysis, the column was uncoupled from the system. In this LC/MSD system, an 1100 binary pump was connected to a carrier or two mobile phases [M1, methanol-water (50:50, v/v); M2, methanol-2-propanol (80:20, v/v)] that was eluted at a flow rate of 0.2 ml/min. The mobile phases were programmed consecutively as follows: a linear gradient of M1 100–20% (M2 0–80%) between 0 and 5 min, a linear gradient of M1 20–0% (M2 80–100%) between 5 and 20 min, an isocratic elution of M1 0% (M2 100%) for 25 min, and an isocratic elution of M1 100% (M2 0%) from 45.1 to 60 min for column equilibrium (a total run time of 60 min). The injection volume was 20 μl each of authentic solutions and hair lipid solutions. The column temperature was maintained at 40°C. The scan measurements using negative and positive ESI in the mass spectrometer were performed with the following settings: heater temperature of nitrogen gas, 350°C; flow of heated dry nitrogen gas, 8.0 l/min; nebulizer gas pressure, 30 p.s.i.; capillary voltage, −3,000 V for negative and 3,000 V for positive; scan range, *m/z* 100–1,000; scan time; 1.98 s. In contrast, the scan measurements using negative and positive APCI in the mass spectrometer were performed with the following settings: vaporizer temperature, 300°C; flow of heated dry nitrogen gas, 5.0 l/min; nebulizer gas pressure, 40 p.s.i.; corona current, 4.0 μA; fragmenter voltage, 200 V; the other settings were the same as for the ESI. These parameters were optimized in preliminary experiments to get the highest abundance of the targeted molecular-related ions for authentic N16DS18. Under optimized conditions of RPLC-APCI-MS, fragmenter voltages of 200 V in channel 1 and 350 V in channel 2 were used.

RPLC-ESI-tandem mass spectrometry analysis

A triple quadrupole API-4000 mass spectrometer was equipped with an ion spray source and Analyst version 1.0 software (Applied Biosystems, Foster City, CA) and interfaced with an Agilent 1100 liquid chromatograph and an 1100 well plate autosampler (Agilent). The RPLC conditions were the same as those for RPLC-APCI-MS. Negative ESI-tandem mass spectrometry (MS/MS) measurements were made, based on the selection of the targeted precursor ions at the peaks detected in channel 1 and identified as CERs by RPLC-APCI-MS, using Q_1 with a scan range of *m/z* 500–850 every 1.0 s, followed by CID in Q_2, followed by detection of the product ion spectra in Q_3 with a scan range of *m/z* 200–500 every 1.0 s. Negative ESI-MS/MS conditions were as follows: ion spray voltage, −3,500 V; ion source heater temperature, 500°C; ion source gases (N_2), 50 p.s.i. for channel 1 and 80 p.s.i. for channel 2; curtain gas (N_2), 20 p.s.i.; declustering potential, −150 V; collision gas (N_2) setting, 5; collision energy, 50 V; collision exit energy, −10 V.

RESULTS

Optimization of LC-MS conditions for CERs

To determine optimized ionization conditions for MS, authentic samples of N16DS18, N24DS18, N16S18, and N24S18 were applied to flow injection MS analysis. When subjected to negative ESI, all authentic CERs tested yielded a prominent deprotonated ion $[M - H]^-$: *m/z* 538 for N16DS18, 650 for N24DS18, 536 for N16S18, and 648 for N24S18. The intensities of the ions in N24DS18 and N24S18 were ∼20% of those in N16DS18 and N16S18, respectively. Upon positive ESI, the authentic NDSs predominantly yielded a protonated adduct ion $[M + H]^+$ and a sodium adduct ion $[M + Na]^+$: *m/z* 540 and 562 for N16DS18 and *m/z* 652 and 674 for N24DS18, respectively; the authentic NSs yielded a protonated adduct and one water loss ion $[M + H - H_2O]^+$ and a sodium adduct ion $[M + Na]^+$: *m/z* 520 and 560 for N16S18 and *m/z* 632 and 672 for N24S18, respectively. The intensities of the ions $[M + H]^+$ in N24DS18 and $[M + H - H_2O]^+$ in N24S18 were ∼30% of those in N16DS18 and N16S18, respectively. On the other hand, when applied to negative APCI, all authentic CERs tested yielded a prominent $[M - H]^-$, similar to negative ESI. The intensities of the ions in N24DS18 and N24S18 were ∼70% of those in N16DS18 and N16S18, respectively. When positive APCI was used, a predominant $[M + H]^+$ was observed in the authentic NDSs and a predominant $[M + H - H_2O]^+$ was observed in the authentic NSs. Little $[M + Na]^+$ was observed. The intensities of the ions $[M + H]^+$ in N24DS18 and $[M + H - H_2O]^+$ in N24S18 were ∼80% of those in N16DS18 or N16S18, respectively. Because positive APCI results in the generation of different kinds of predominant ions between the CER types (NDS and NS) but not much difference in the intensities of ions between shorter (N16) and longer (N24) chains of FAMs, the positive APCI was selected as an ionization condition.

The effects of fragmenter voltage, which induces in-source CID, on mass spectra of authentic CERs in positive APCI were examined to get more informative ions than the molecular-related ions for the identification of CERs. The detected ions were assigned based on previous studies and nomenclature (O = $[M + H - RCO]^+$, O′ = $[M + H - RCO - H_2O]^+$, O″ = $[M + H - RCO - 2H_2O]^+$, U = $[RCONH_3]^+$) (37, 47). **Figure 1** presents mass spectra of N16DS18 and N16S18 with different fragmenter voltages. N16DS18 yielded $[M + H]^+$ = 540 and $[M + H - H_2O]^+$ = 522 at the fragmenter voltage of 200 V (Fig. 1A), $[M + H]^+$, $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$ = 504, O = 302, O′ = 284, O″ = 266, and U = 256 at 300 V (Fig. 1B), and $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$, and O′, O″, U, and $[M + H - C_{15}H_{31}CO - H_2O - HCHO]^+$ = 254 at 400 V (Fig. 1C). Because deacyl ions, such as the O, O′, and O″, are informative for constitutional SPM, they were regarded as SPM-related ions. Under the higher voltage, water loss ions and SPM-related ions of N16DS18 were readily produced. The mass spectra of N24DS18 were almost the same as those of N16DS18 (data not shown). In contrast, N16S18 yielded similar kinds of ions to those of N16DS18, except for undetectable $[M + H]^+$, detectable $[M + H - H_2O - HCHO]^+$ = 490, undetectable O, undetectable U, and detectable $[M + H - C_{15}H_{31}CO - H_2O - HCHO]^+$ at 300 V, and undetectable $[M + H - H_2O]^+$, undetectable $[M + H - 2H_2O]^+$, and undetectable U at 400 V (Fig. 1D–F). Compared with N16DS18, N16S18 had the characteristics of a higher production of water loss ions and SPM-related ions as well as the production of formaldehyde loss ions. The mass spectra of N24S18 were almost the same as those of N16S18 (data not shown). The relationships between the observed predominant ions and

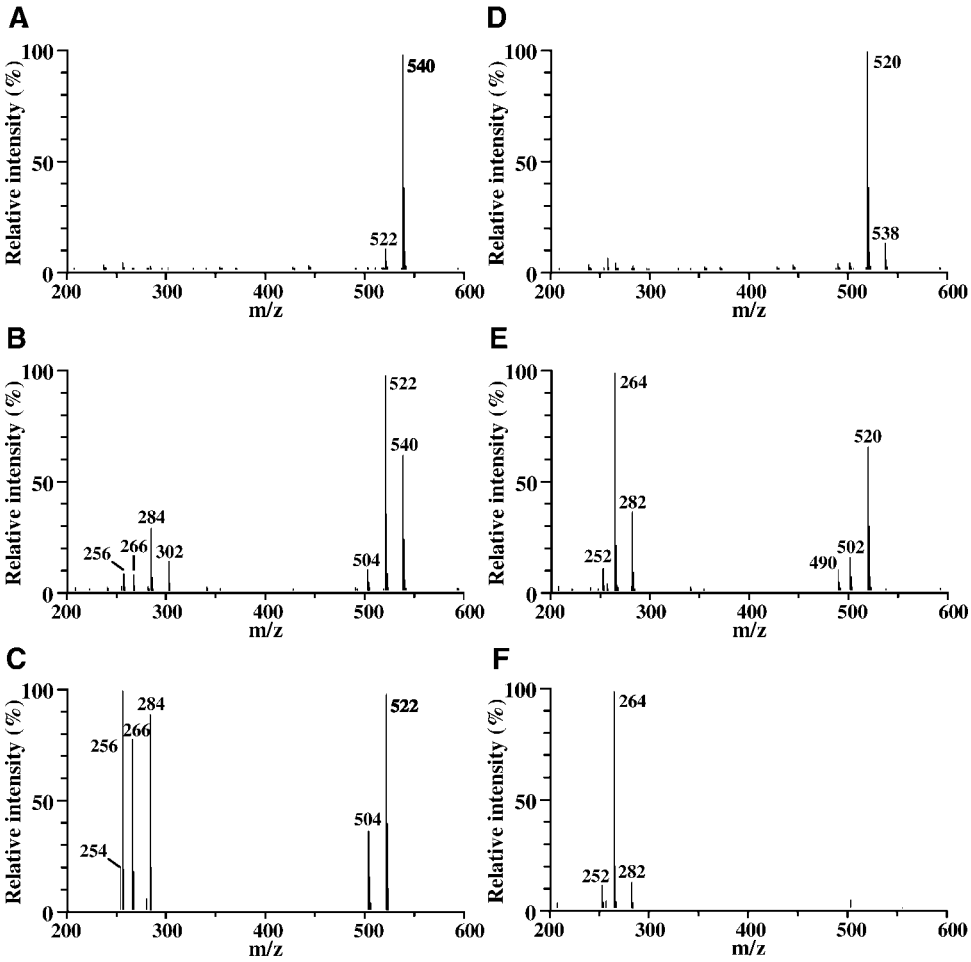

Fig. 1. Positive atmospheric pressure chemical ionization (APCI) mass spectra of N16DS18 and N16S18 in flow injection mass spectrometry (MS) analysis. Fragmenter voltages were 200 V (A), 300 V (B), and 400 V (C) for N16DS18 and 200 V (D), 300 V (E), and 400 V (F) for N16S18 at a concentration of 10 μg/ml. Experimental conditions were as follows: injection volume, 20 μl; carrier, methanol at a flow rate of 0.2 ml/min; vaporizer temperature, 300°C; heater temperature of nitrogen gas, 350°C; flow rate of heated dry nitrogen gas, 5.0 l/min; nebulizer gas pressure, 40 p.s.i.; capillary voltage, 3,000 V; corona current, 4.0 μA; scan range, *m/z* 100–1,000 (only 200–600 is shown here).

tested fragmenter voltages are shown in **Fig. 2**. In the observable ions in the authentic NDSs at all voltages tested, the intensities of $[M + H]^+$ were higher than those of $[M + H - H_2O]^+$ and exhibited maxima in the vicinity of 200 V, whereas the intensities of O′ were higher than those of O″ and exhibited maxima in the vicinity of 350 V (Fig. 2A, B). The authentic NSs had almost similar relationships to NDSs except that the kinds of intensive ions were different. Thus, in the NSs, the predominant molecular-related ions $[M + H - H_2O]^+$ had maximal intensities at 200–250 V, whereas the predominant SPM-related ions O″ had maximal intensities at 300–350 V. This suggested that in-source CID with different fragmenter voltages upon positive APCI-MS is effective at identifying CERs, including isobaric CERs, because 200 and 350 V provide information on the molecular weights and constitutional SPMs, respectively.

Based on these results, positive APCI-MS was used with two channel detections at fragmenter voltages of 200 V in channel 1 and 350 V in channel 2. As for RPLC conditions, the gradient elution described in Materials and Methods was adopted to comprehensively separate and detect CERs with wide-chain distributions in FAMs. Total ion chromatograms (TICs) of a mixture of the four authentic CERs by this RPLC-APCI-MS are shown in **Fig. 3A, B**. From the two TICs, first multimass chromatograms consisting of *m/z* 540, 652, 520, and 632 for $[M + H]^+$ of N16DS16, $[M + H]^+$ of N24DS16, $[M + H - H_2O]^+$ of N16S18, and $[M + H - H_2O]^+$ of N24S18 in channel 1, respectively, and second multimass chromatograms consisting of *m/z* 284 and 264 for ion O′ of the NDSs and ion O″ of the NSs in channel 2, respectively, were constructed (Fig. 3C, D). It was confirmed that all peaks detected as molecular-related ions in channel 1 have identical retention times (RTs) to those detected as SPM-related ions in channel 2. This indicated that the combined information, consisting of molecular-related ions in channel 1, SPM-related ions in channel 2, and RTs in both channels, enables us to com-

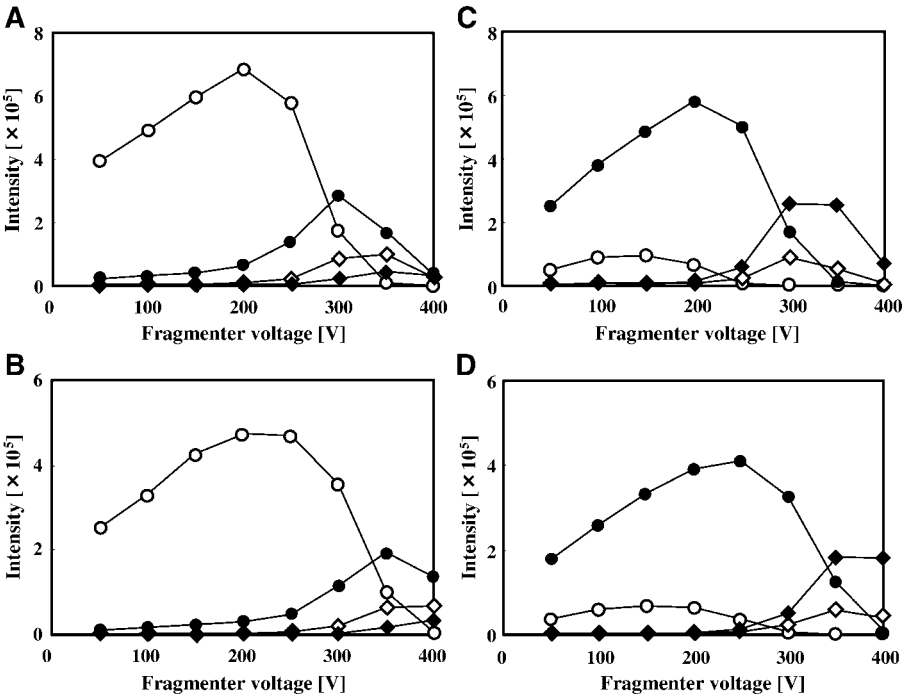

Fig. 2. Effect of fragmenter voltages on intensities of ions derived from authentic N16DS18 (A), N24DS18 (B), N16S18 (C), and N24S18 (D) at a concentration of 10 μg/ml in flow injection MS analysis using positive APCI. Open circles, $[M + H]^+$; closed circles, $[M + H - H_2O]^+$; open diamonds, $[M + H - RCO - H_2O]^+$; closed diamonds, $[M + H - RCO - 2H_2O]^+$. For experimental conditions, see Fig. 1 legend, except for fragmenter voltages of 0–400 V.

prehensively identify various CER molecules even if isobaric CERs are included.

Identification and profiling of CER molecules in human hair

Figure 4 shows RPLC-APCI-MS TICs of lipid extracts prepared from Hair-A. Using the TICs, all possible combinations between the FAMs and the SPMs within CER types belonging to NDSs, NSs, ADSs, and ASs (23, 24) were investigated. For example, if N16DS18 is present, a detected peak in a mass chromatogram of *m/z* 540 ($[M + H]^+$) extracted from the TIC of channel 1 should have an identical RT to that of a detected peak in *m/z* 284 (ion O′) from channel 2. Similarly, if N16S18 is present, the RT of a detected peak in a mass chromatogram of *m/z* 520 ($[M + H - H_2O]^+$) must be in accord with that in *m/z* 264 (ion O″). In this method, using 20 μl injections, the limit of detection was ~2 and 10 ng of authentic N16DS18 for channels 1 and 2, respectively, and ~2 and 5 ng of N16S18 (data not shown). Therefore, >10 ng of NDSs and >5 ng of NSs in the injected solution seemed to be targeted for CERs of Hair-A.

RPLC-APCI-MS multimass chromatograms of CERs, extracted from the TICs shown in Fig. 4, in Hair-A are shown in **Fig. 5**, where peaks 1–48 in channel 1 are indicated as CER molecules belonging to NDSs, NSs, ADSs, and ASs. Those peaks were identified based on the molecular weights from molecular-related ions in channel 1, the constitutional SPMs from SPM-related ions in channel 2, and their identical RTs in both channels 1 and 2. **Table 1** lists CER molecules identified in hair. Major FAMs with or without a hydroxyl group contained saturated chains with even numbers of carbon atoms, whereas some FAMs containing saturated or unsaturated chains with odd numbers of carbon atoms were also found. The major SPM was a DS18 moiety; DS17 and DS19 moieties with odd numbers of carbon atoms were also detected, together with S18, DS20, and DS16. The detection of more than two SPM-related ions in channel 2 at the identical RT of one molecular-related ion detected in channel 1 indicated that there are several groups that have identical molecular weights but different combinations of FAMs and SPMs, such as N20DS18 and N18DS20. Furthermore, in the case of some odd carbon atom-containing CER molecules, twin peaks of identical CER molecules were observed, as represented in peaks 30 and 32 (N23DS18 + N22DS19). As a result, 73 species of CER molecules in hair were comprehensively profiled (Fig. 5).

To verify the validity of the identification of CER molecules using this RPLC-APCI-MS, product ion analyses using RPLC-negative ESI-MS/MS were conducted. Because negative ESI-MS/MS provides the FAM-related ions of CER molecules (36, 39), it was expected that this would be complementary to the RPLC-APCI-MS, providing information on the constitutional SPMs. Under the same LC conditions as the RPLC-APCI-MS, the deprotonated ions $[M - H]^-$ with substantially identical RTs to RPLC-APCI-MS were analyzed as precursor ions. A product ion spec-

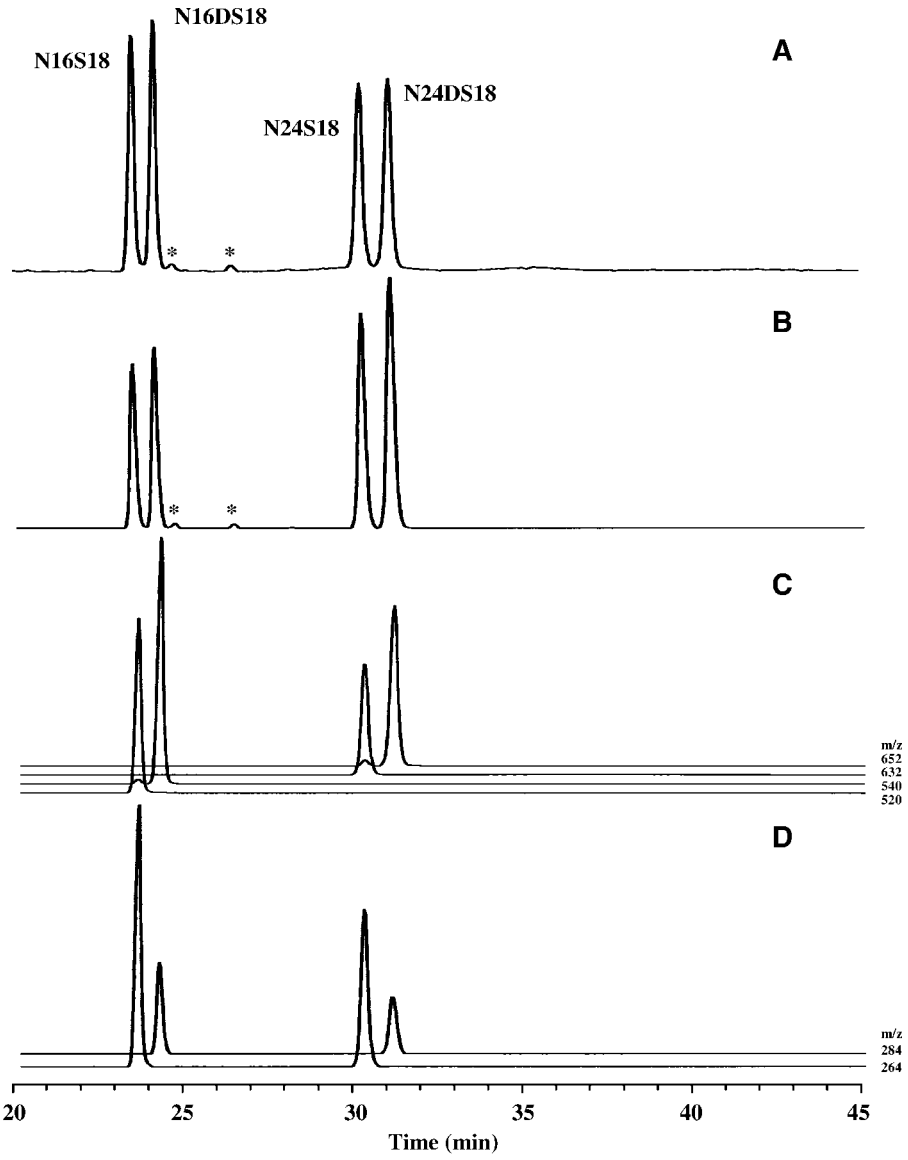

Fig. 3. Reverse-phase liquid chromatography (RPLC)-APCI-MS total ion chromatograms (TICs) and multimass chromatograms of authentic ceramides (CERs) at a concentration of 10 μg/ml using two channel detections of fragmenter voltages of 250 V in channel 1 and 350 V in channel 2. A, B: TICs in channels 1 and 2, respectively. C, D: Multimass chromatograms in channels 1 and 2, respectively. Asterisks indicate unidentified substances that originate from authentic N24DS18. Experimental conditions were as follows: L-column ODS, 2.1 mm × 150 mm; column temperature, 35°C; mobile phases M1, methanol-water (50: 50, v/v) and M2, methanol-2-isopropanol (80:20, v/v); flow rate, 0.2 ml/min; injection volume, 20 μl. For gradient elution, see Materials and Methods; for other settings, see Fig. 1 legend.

trum of authentic N16DS18 is shown in **Fig. 6A**, where fragment ions *m/z* 280, 237, 296, and 254 in decreasing order were obtained from the precursor ion *m/z* 538. These ions (*m/z* 280, 237, 296, and 254) were assigned to T [CH2 = CH − NHCO = $C_{15}H_{30}]^-$, V [CHO = $C_{15}H_{28}]^-$, S [CHO − CH_2 − NHCO = $C_{15}H_{30}]^-$, and U [NHCO − $C_{15}H_{31}]^-$, respectively, according to Ann and Adams (48). Based on this assignment of FAM-related ions, all precursor ions of interest were structurally analyzed. Figure 6B shows a product ion spectrum from precursor ion *m/z* 594, identified as ND20DS18 and N18DS20 in the RPLC-APCI-MS (peak 19 in Fig. 5). In this spectrum, *m/z* 336, 293, and 352 and *m/z* 308, 265, and 324 were assigned to the T, V, and S of N20DS18 and the T, V, and S of N18DS20, respectively. Thus, the results by RPLC-ESI-MS/MS agreed with those by RPLC-APCI-MS. Figure 6C, D show spectra from precursor ion *m/z* 636, which was eluted in the RPLC-APCI-MS as a twin peak (peaks 30 and 32 in Fig. 5) and identified as ND23DS18 and N22DS19. In both peaks, ions *m/z* 378, 335, 394, and 353 and *m/z* 364, 321, and 380 were assigned to the T, V, S, and U of N23DS18 and the T, V, and S of N22DS19, respectively. These results also agreed with the identification by RPLC-APCI-MS. Such RPLC-ESI-MS/MS analysis corroborated the presence of all 73 spe-

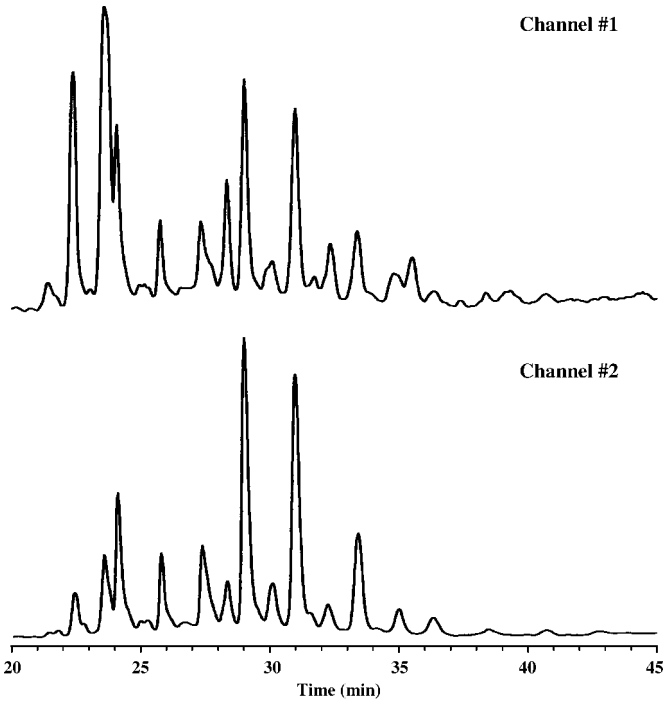

Fig. 4. RPLC-APCI-MS TICs of lipid extracts prepared from hair sample Hair-A. For experimental conditions, see Fig. 3 legend.

cies of CER molecules in hair, as listed in Table 1. This demonstrates that isobaric and isomeric CER molecules are truly present in hair.

Applications

The RPLC-APCI-MS method was applied to the analysis of CER molecules in hair fibers collected from three different females (Hair-B, Hair-C, and Hair-D). The analysis revealed that all hair samples tested have the same 73 CER molecules as those identified in Hair-A (Table 1). N24DS18 (+N22DS20), N24:1DS18, N16DS18 (+N18DS16), N22DS18 (+N20DS20), N26DS18 (+N24DS20), N18DS18, A16DS18, and N20DS18 (+N18DS20) were commonly abundant in all samples tested. However, there seemed to be variations in the peak heights among the three different sets of multimass chromatograms derived from the following CER molecules: A16S18, N16S18, N16DS18 (+N18DS16), N18:1DS18, N20DS18 (+N18DS20), N24:1DS18, N26:1DS18 (+N24:1DS20), and N26DS18 (+N24DS20) (data not shown).

Figure 7 shows RPLC-APCI-MS multimass channel 1 chromatograms of CERs in Hair-Eb and Hair-Eg, which were black and gray hair fibers collected from a male. All 73 species of CER molecules, as listed in Table 1, were observed in the black and gray hairs, in common with Hair-A to Hair-D. In addition, the kinds of abundant CER molecules in Hair-Eb and Hair-Eg completely agreed with those in Hair-B to Hair-D. By comparison between the two different sets of multimass chromatograms, it was observed that some peaks of saturated FAM-containing NDSs, such as N18DS18, N20DS18 (+N18DS20), N22DS18 (+N20DS20), and N26DS18 (N24DS20), were smaller in Hair-Eb than in Hair-Eg (asterisks in Fig. 7), whereas other peaks derived from N24:1DS18 and N26:1DS18 (+N24:1DS20) were larger in Hair-Eb (double asterisks in Fig. 7).

DISCUSSION

The aim of this study was to develop a simple method for the comprehensive profiling of mixed CER molecules and to comprehensively identify CER molecules in hair. First, by examination of authentic CERs, we selected the positive APCI characteristic for the detection of predominant molecular-related ions between NDSs and NSs and for slight differences in their intensities among different CER molecules. The former characteristic is useful for discriminating between a NDS molecule with an unsaturated FAM and a NS molecule with a saturated FAM, even though they are of identical molecular weights. The latter characteristic is favorable for the comprehensive profiling of CERs, because the sizes of peaks observed in the chromatogram are directly comparable among different CER molecules. Next, we adopted two channel detections using two different fragmenter voltages (200 and 350 V) that determine in-source CID and then provide SPM-related ions in 350 V as well as molecular-related ions in 200 V. This enables the identification of CER molecules, that is, various combinations of FAMs and SPMs. Furthermore, we connected this APCI-MS to RPLC, which provides RTs for the hydrophobicity of CER molecules as additional information. Therefore, we can conclude that we have successfully developed a new method, RPLC-APCI-MS, for the comprehensive identification and profiling of all 73 CER molecules, based on molecular weights, constitutional SPMs, and RTs.

However, in this method, we also have to take into consideration some potential artifacts. For example, there is a possibility of overlap between the U and O′ (m/z 256) derived from N16DS18 and N18DS16, respectively. Inversely, there is another possibility of overlap between the O′ and U (m/z 284) derived from N16DS18 and N18DS16, respectively. Thus, two isobaric CER molecules that have a difference in two carbon atoms of FAMs and SPMs yield identical ions, as a result of their mass spectral characteristics. In such cases, however, we can check the presence of a peak corresponding to ion O′′ derived from a tentatively identified CER molecule as a peak of multimass chromatograms at m/z 238 for N18DS16. Actually, checks of the original data in Fig. 5 demonstrated that all of the identifications shown in Table 1 are correct. Furthermore, as seen in N16DS18 and N18DS16, the O″ (m/z 266) derived from N16DS18 possibly overlaps with the identical $[C_{17}H_{35}CONH_3 - H_2O]^+$ from N18DS16. However, ion $[C_{15}H_{31}CONH_3 - H_2O]^+$ from authentic N16DS18 was not observed in its mass spectrum (Fig. 1C), which indicates that this potential artifact can be ignored. The accuracy of our identification is also corroborated by structural analysis using RPLC-ESI-MS/MS in the negative ion mode, based on a different principle from RPLC-APCI-MS.

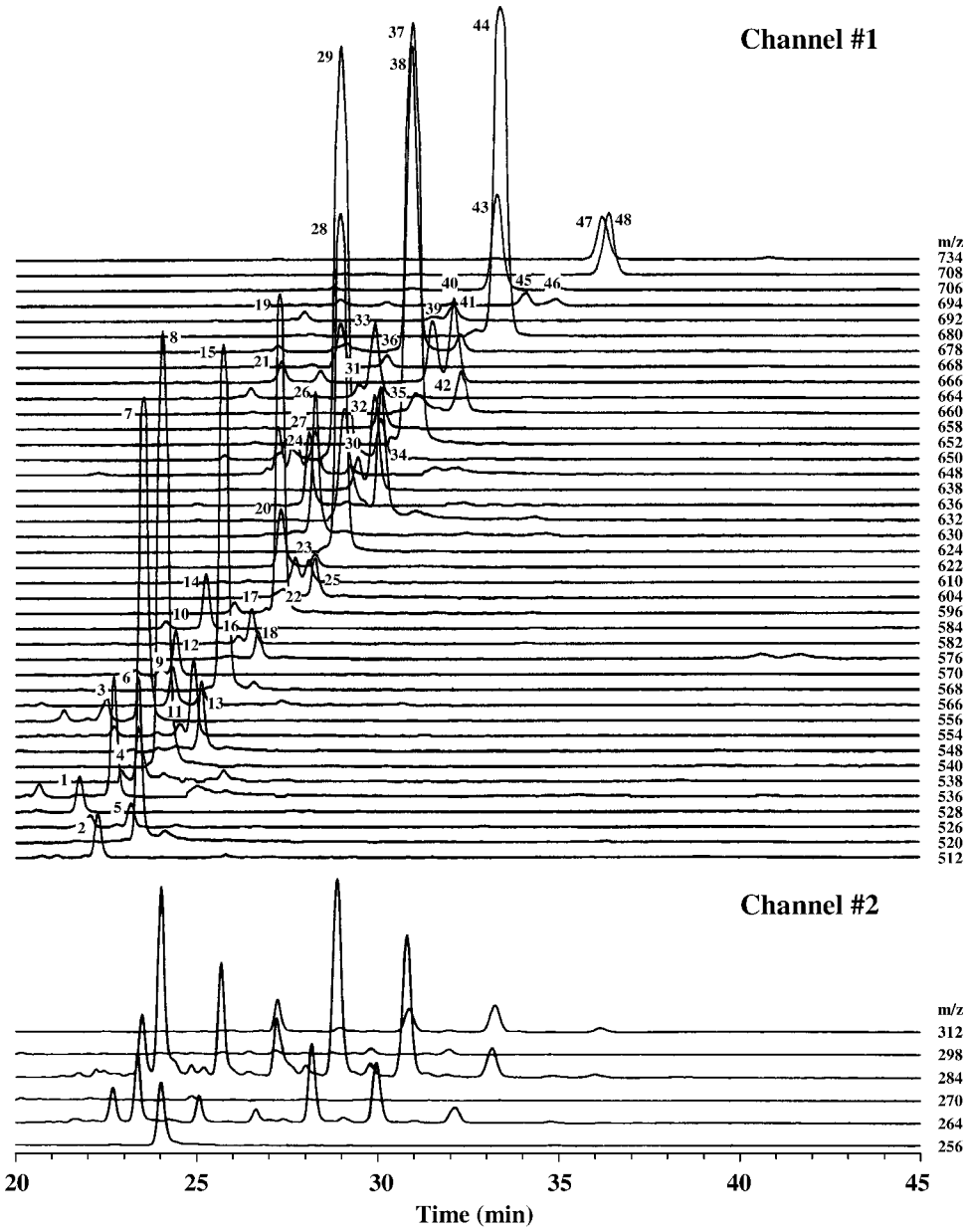

Fig. 5. RPLC-APCI-MS multimass chromatograms of CER molecules, extracted from TICs shown in Fig. 4, in hair sample Hair-A. For peaks, see Table 1; for experimental conditions, see Fig. 3 legend.

The use of RPLC-MS with positive APCI in this study is not the first for the analysis of CERs; Couch et al. (31) previously reported RPLC-APCI-MS for their analysis in human cells. However, their RPLC-APCI-MS technique did not allow the comprehensive profiling of CER molecules. Although Lee, Lee, and Yoo (39) used a cone voltages switching method for the structural analysis of CERs for cosmetic raw materials with RPLC-ESI-MS, they used only mass spectra of eluted peaks and not the multimass chromatograms that enable comprehensive profiling. Therefore, it is worth noting that our RPLC-APCI-MS technique is new with regard to the use of multimass chromatograms that are produced by two channel detections under two different in-source CID conditions, which makes possible the comprehensive profiling of all CER molecules in hair. On the other hand, many profiling methods for CER molecules using MS have been reported (30, 31, 35, 38, 41, 42, 44, 45). Among those, the method using direct MS/MS and neutral loss ion scan described by Han (38) is superior to the others because it can simultaneously monitor a set of CER molecules having a given SPM with high sensitivity. However, the monitored CER molecules are restricted to those that can be expected in advance, as a result of the requirement of setting for a neutral loss ion before the analysis. Another method uses normal-phase LC-APCI-MS for extremely complex mixtures of CER molecules (41, 45) and is available for the simultaneous profiling of CER molecules for each CER type. However, based on this principle, the method is useful for the patterning of CERs but cannot identify combinations between

TABLE 1. CER molecules identified in hair

Peak[a]	Identification	RPLC-Electrospray Ionization-MS/MS Retention Time	Ion 1[b]	Ion 2[c]	RPLC-Atmospheric Pressure Chemical Ionization-MS Ion 3[d]	Ion 4[e]	Ion 5[f]
		min			*m/z*		
1	A14DS18	21.9	528	284	526	268	225
2	N14DS18	22.4	512	284	510	252	209
3	A16S18	22.8	536	264	552	296	253
4	N16:1DS18	23.0	538	284	536	278	235
5	N15DS18	23.3	526	284	524	266	223
6	N16S18	23.5	520	264	536	280	237
7	A16DS18	23.6	556	284	554	296	253
8	N16DS18	24.1	540	284	538	280	237
	N18DS16	24.1	540	256	538	308	265
9	N18:1DS18	24.4	566	284	564	306	263
10	A17DS18	24.5	570	284	568	310	267
11	N17DS18[g]	24.7	554	284	552	294	251
12	N17DS18[h]	25.0	554	284	552	294	251
	N18DS17[h]	25.0	554	270	552	308	265
	N16DS19[h]	25.0	554	298	552	280	237
13	N18S18	25.2	548	264	564	308	265
14	A18DS18	25.3	584	284	582	324	281
15	N18DS18	25.8	568	284	566	308	265
16	N19DS18[g]	26.3	582	284	580	322	279
17	N19DS18[h]	26.6	582	284	580	322	279
	N18DS19[h]	26.6	582	298	580	308	265
18	N20S18	26.8	576	264	592	336	293
19	N20DS18	27.4	596	284	594	336	293
	N18DS20	27.4	596	312	594	308	265
20	N22:1DS18	27.4	622	284	620	362	319
21	A24:1DS18	27.4	666	284	664	406	363
22	N21DS18[g]	27.8	610	284	608	350	307
23	N21DS18[h]	28.2	610	284	608	350	301
	N20DS19[h]	28.2	610	298	608	336	293
24	N23:1DS18	28.2	636	284	634	376	333
25	N22S18	28.4	604	264	620	364	321
26	N24:1S18	28.4	630	264	646	390	347
27	A24S18	28.4	648	264	664	408	365
28	N22DS18	29.0	624	284	622	364	321
	N20DS20	29.0	624	312	622	336	293
29	N24:1DS18	29.1	650	284	648	390	347
30	N23DS18[g]	29.5	638	284	636	378	335
	N22DS19[g]	29.5	638	298	636	364	321
31	N25:1DS18[g]	29.5	664	284	662	404	361
32	N23DS18[h]	30.0	638	284	636	378	335
	N22DS19[h]	30.0	638	298	636	364	321
33	N25:1DS18[h]	30.0	664	284	662	404	361
	N24:1DS19[h]	30.0	664	298	662	390	347
34	N24S18	30.2	632	264	648	392	349
35	N26:1S18	30.2	658	264	674	418	375
36	A24DS18	30.3	668	284	666	408	365
	A22DS18	30.3	668	312	666	390	337
37	N24DS18	31.0	652	284	650	392	349
	N22DS20	31.0	652	312	650	364	321
38	N26:1DS18	31.0	678	284	676	418	375
	N24:1DS18	31.0	678	312	676	390	347
39	N25DS18[g]	31.5	666	284	664	406	363
	N24DS19[g]	31.5	666	298	664	392	349
40	N25DS18[h]	32.1	666	284	664	406	363
	N24DS19[h]	32.1	666	298	664	392	349
	N23DS20[h]	32.1	666	312	664	378	335
41	N27:1DS18	32.2	692	284	690	432	389
	N26:1DS19	32.2	692	298	690	418	375
42	N26S18	32.4	660	264	676	420	377
43	N28:1DS18	33.3	706	284	704	446	403
	N26:1DS20	33.3	706	312	704	418	375
44	N26DS18	33.4	680	284	678	420	377
	N24DS20	33.4	680	312	678	392	349
45	N27DS18[g]	34.1	694	284	692	434	391
	N26DS19[g]	34.1	694	298	692	420	377
	N25DS20[g]	34.1	694	312	692	406	363
46	N27DS18[h]	34.8	694	284	692	434	391
	N26DS19[h]	34.8	694	298	692	420	377
	N25DS20[h]	34.8	694	312	692	406	363
47	N30:1DS18	36.2	734	284	732	474	431
	N28:1DS20	36.2	734	312	732	446	403
48	N28DS18	36.4	708	284	706	448	405
	N26DS20	36.4	708	312	706	420	377

CER, ceramide; MS, mass spectrometry; MS/MS, tandem mass spectrometry; RPLC, reversed-phase liquid chromatography.

[a] See Fig. 6.

[b] $[M + H]^+$ for CERs consisting of nonhydroxy fatty acid and dihydrosphingosine moieties (NDSs) and CERs consisting of α-hydroxy fatty acid and dihydrosphingosine moieties (ADSs), or $[M + H - H_2O]^+$ for CERs consisting of nonhydroxy fatty acid and sphingosine moieties (NSs) and CERs consisting of α-hydroxy fatty acid and sphingosine moieties (ASs).

[c] Ion O′ (37, 47) for NDSs and ADSs, or ion O″ (37, 47) for NSs and ASs.

[d] $[M - H]^-$ in Q1 of MS/MS.

[e] Ion T (48) in Q3 of MS/MS.

[f] Ion V (48) in Q3 of MS/MS.

[g] Tentatively identified as a CER molecule with a branched chain.

[h] Tentatively identified as a CER molecule with two straight chains.

FAMs and SPMs. Therefore, neither of those methods, nor the others, are useful for comprehensive profiling. In contrast to those techniques, our method enables the comprehensive identification and profiling of all CERs with various combinations of FAMs and SPMs. Furthermore, as described below, our method is also superior because the RTs can reveal the presence of isomeric CER molecules that would not be expected at all in advance. It should be noted that such comprehensive profiling is absolutely impossible by direct MS/MS and may be impossible by normal-phase LC-MS. Therefore, we conclude that our method based on molecular-related ions, SPM-related ions, and RTs in multimass chromatograms can comprehensively profile CER molecules.

In this study, we have shown that there are 73 kinds of CER molecules belonging to NDSs, NSs, ADSs, and ASs and that NDSs are predominant in hair. These characteristics are common in all tested samples collected from four different females and one male, although some of them may be variable among the four different hairs and between black and gray hairs from one male. Previous reports (23, 24) described 25 CER molecules in hair. In this study, all of those CER molecules, except for N14S18, were detected. This implies that we have discovered 49 new species of CER molecules in hair. Some of them might be described for the first time in humans. The predominant CERs in human cells/tissues are S18-containing NSs with less abundant S18-containing ASs (10–15), whereas the CERs in human stratum corneum are composed of S, phytosphingosine, and 6-hydroxy-sphingosine (21), with less abundant DS. We do not apply our method to the analysis of stratum corneum CERs because it is impossible

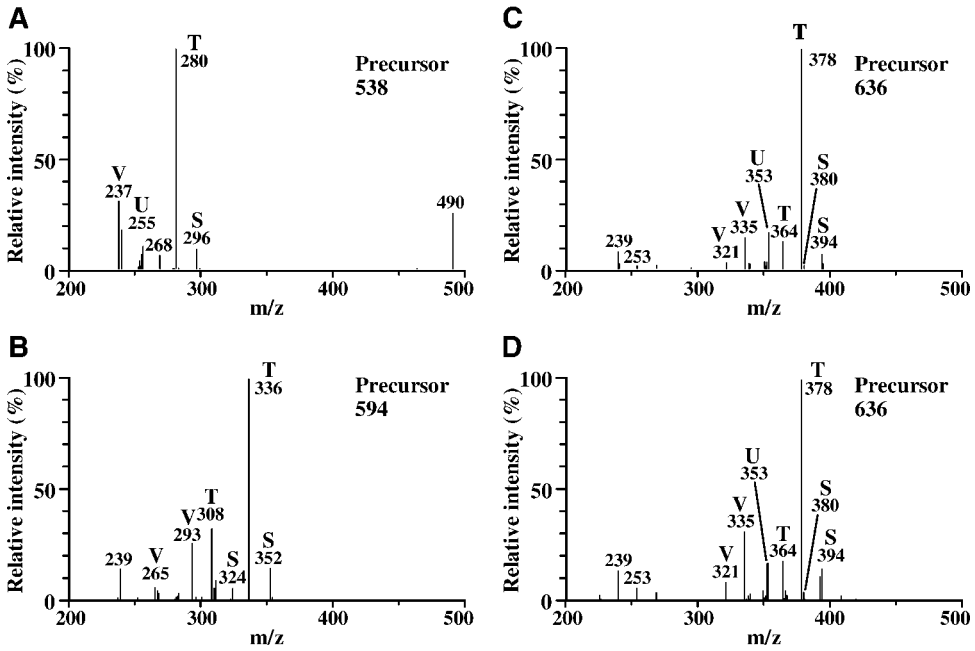

Fig. 6. Product ion spectra of 10 μg/ml N16DS18 (A), peak 19 (B), peak 30 (C), and peak 32 (D) by RPLC-electrospray ionization (ESI)-tandem mass spectrometry (MS/MS) in the negative ion mode. T = [CH2 = CH − NHCO = $C_{15}H_{30}]^-$, V = [CHO = $C_{15}H_{28}]^-$, S = [CHO − CH_2 − NHCO = $C_{15}H_{30}]^-$, and U = [NHCO − $C_{15}H_{31}]^-$. For experimental conditions for liquid chromatography, see Fig. 3 legend. Experimental conditions for ESI-MS/MS were as follows: scan range, *m/z* 500–850 every 1.0 s for Q1 and *m/z* 200–500 every 1.0 s for Q3; ion spray voltage, −3,500 V; collision energy, 50 V in Q2. For other parameters, see Materials and Methods.

to comprehensively profile extremely complex CERs of human stratum corneum. Farwanah et al. (49) recently reported their molecular species analyzed by normal LC-APCI-MS, with no structural analysis of them, in which NSs, NDSs, and ASs are thought to have fatty acids with 24–34, 24–32, and 15–18 carbon atoms based on the assumption that all CERs have sphingoids with 18 carbon atoms. No kinds of ADSs were detected. In contrast, CER molecules in hair are characterized by the predominant NDSs and ADSs with less abundant NSs and ASs, as in previous studies (23, 24). In addition, the present study has revealed other unique characteristics by the detailed analysis of their FAMs and SPMs. The first characteristic is that hair contains many unsaturated FAM-containing CER molecules, present as N16:1, N18:1, N22:1, N23:1, A24:1, N24:1, N25:1, N26:1, N27:1, N28:1, and N30:1 (Table 1), compared with other human cells/tissues. The second characteristic is that hair contains many isobaric CER molecules, as found in those with identical molecular weights but different FAMs and SPMs (Table 1). The third characteristic is that hair contains not only even carbon atom-containing CER molecules but also odd carbon atom-containing CERs, in which either FAM or SPM has an odd number of carbon atoms.

Those odd-CER molecules are unique. Each of them frequently forms an isobaric group together with other odd-CER molecule(s). The odd-SPMs, such as DS17 and DS19, are included only in the odd-CERs. Although the presence of DS17 and DS19 was described previously in human stratum corneum (50), they were found only in hydrolysates of CERs. Therefore, the direct detection of DS17 and DS19 from intact CER molecules in this study is the first in all CER studies in humans. Thus, the odd-SPM is included only in odd-CER, whereas the odd-CERs frequently form a twin peak whose FAMs and SPMs are identical to each other by RPLC. The paired peaks may be responsible for the isomeric configurations that influence the hydrophobic properties of CER molecules, and we assume that the earlier eluted odd-CER(s) in the RPLC might contain a branched chain and the later eluted odd-CER(s) might contain two straight chains. This assumption is based on our experience, such as with 18-methyl eicosanoic acid (branched C_{21}), which elutes earlier than heneicosanoic acid (straight C_{21}) on RPLC (47). Actually, it is known that there is an odd-SPM with a branched chain in mammals (14). The presence of such odd-CERs in hair raises question about their biosynthesis, because this might indicate the possibility of new de novo pathways and the regulation of CER metabolism in humans (14). However, attention should be paid to this assumption, because there is little evidence regarding the presence of odd-CER molecules with branched chains.

The newly developed RPLC-APCI-MS method is available for the qualitative analysis of all CER molecules in hair, as demonstrated here. However, this method is not universal. It is not appropriate for the quantitative analysis of CER molecules, although the profiles of multimass chromatograms may approximately reflect their levels.

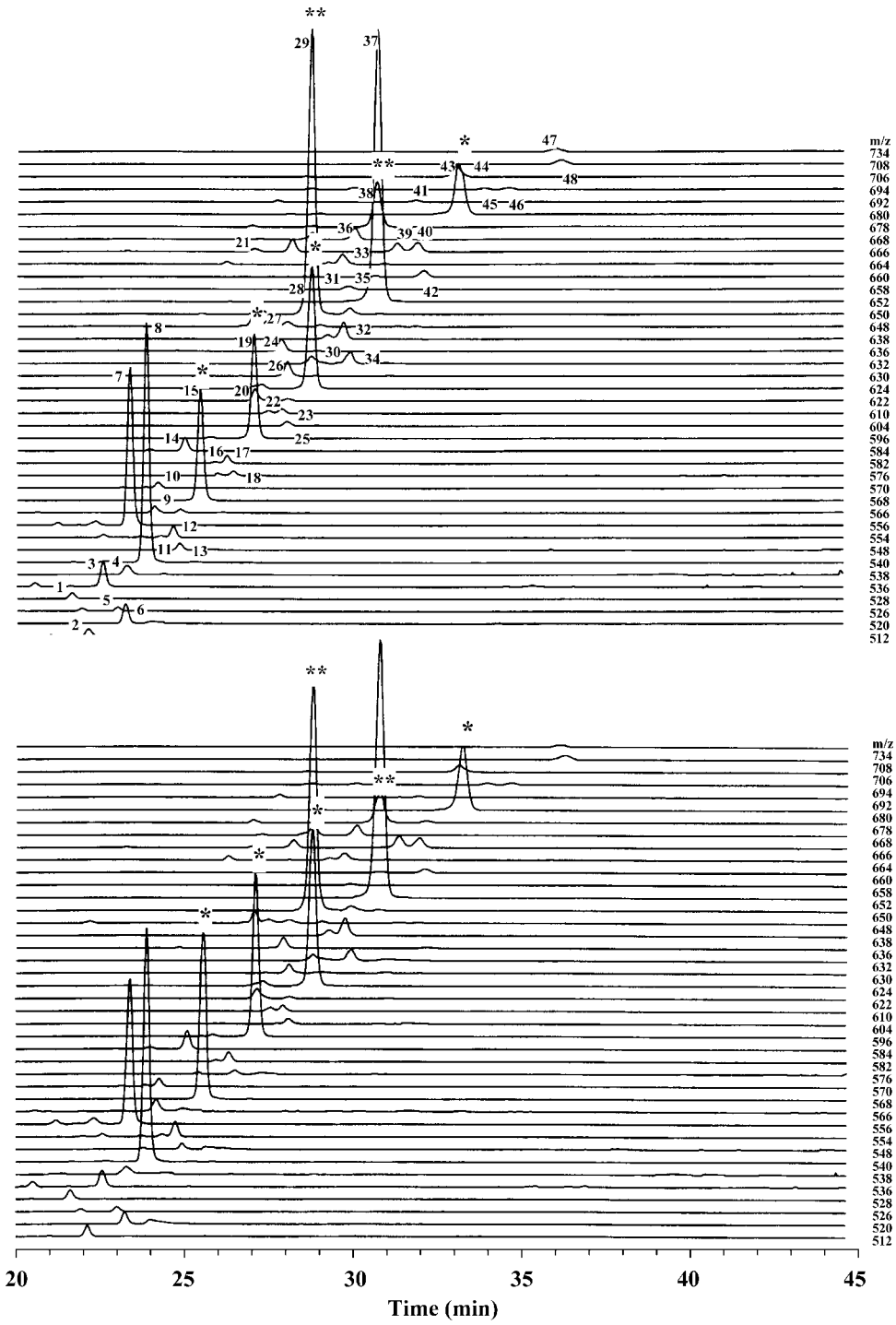

Fig. 7. RPLC-APCI-MS multimass channel 1 chromatograms of CER molecules in black (top) and gray (bottom) hair samples Hair-Eb and Hair-Eg, respectively. For asterisks and double asterisks, see text; for peaks, see Table 1; for experimental conditions, see Fig. 3 legend.

There is another reason that this method is not useful for the trace analysis of CERs in small hair samples: it does not have high sensitivity, as shown in the limit of detection being ~10 and 5 ng of authentic N16DS18 and N16S18, respectively. We need to develop a highly sensitive method for the comprehensive determination of the CERs to accurately pursue their metabolic processes, such as the conversion of NDSs to NSs and the occurrence of odd-CERs, during keratinization at regions near the hair follicle. This will be our future work.

In conclusion, we have developed a method using RPLC-APCI-MS for the comprehensive profiling of CER molecules and have analyzed CER molecules in hair. Potential artifacts of this method must be kept in mind. Because this method is based on the simultaneous detection of molecular-related ions and SPM-related ions, the

comprehensive identification and profiling of CER molecules, including isobaric CERs, is possible. The RTs in RPLC offer information on the hydrophobic properties of CER molecules that also enables the discriminative detection of isomeric CERs, which are assumed to include CER molecules with branched chains in FAMs or SPMs. This application has revealed 73 kinds of CER molecules in hair, of which 49 CER molecules are novel. These 73 CER molecules are characterized by unsaturated FAMs, isobaric CERs, unusual odd-SPMs such as DS17 and DS19, and isomeric CERs. This profile of the CERs qualitatively seems to be common in different individuals and different hair fibers, such as black and gray ones, collected from a male, despite their levels seeming to be different. The newly developed RPLC-ESI-MS is a powerful tool for the investigation of physicochemical and physiological roles of CER molecules in hair.

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