methods

Comprehensive quantification of ceramide species in human stratum corneum[S]

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Abstract One of the key challenges in lipidomics is to quantify lipidomes of interest, as it is practically impossible to collect all authentic materials covering the targeted lipidomes. For diverse ceramides (CER) in human stratum corneum (SC) that play important physicochemical roles in the skin, we developed a novel method for quantification of the overall CER species by improving our previously reported profiling technique using normal-phase liquid chromatography-electrospray ionization-mass spectrometry (NPLC-ESI-MS). The use of simultaneous selected ion monitoring measurement of as many as 182 kinds of molecular-related ions enables the highly sensitive detection of the overall CER species, as they can be analyzed in only one SC-stripped tape as small as 5 mm × 10 mm. To comprehensively quantify CERs, including those not available as authentic species, we designed a procedure to estimate their levels using relative responses of representative authentic species covering the species targeted, considering the systematic error based on intra-/inter-day analyses. The CER levels obtained by this method were comparable to those determined by conventional thin-layer chromatography (TLC), which guarantees the validity of this method. This method opens lipidomics approaches for CERs in the SC.—Masukawa, Y., H. Narita, H. Sato, A. Naoe, N. Kondo, Y. Sugai, T. Oba, R. Homma, J. Ishikawa, Y. Takagi, and T. Kitahara. **Comprehensive quantification of ceramide species in human stratum corneum. *J. Lipid. Res.* 2009.** 50: **1708–1719.**

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To perform lipidomics (1), advanced analytical technologies, such as direct MS and LC-MS, are preferably used (2–10). To characterize differences in lipidomes among biological samples of interest, the relative intensities or areas given from mass spectrometers are only helpful as semi-quantitative values (11, 12). Precise quantitative values of lipidomes of interest would also be desirable, especially in the case of evaluations of lipid dynamics in de novo pathways, such as alterations of some species of the targeted lipidomes into others. Similar to common quantitative analyses using direct MS or LC-MS that need authentic standards together with internal ones, quantitative lipidomics may also require a standard set of all lipid species targeted, because each lipid species has a different molar response in MS detection except for special cases. However, it is practically impossible to obtain all authentic species, including not only different even straight carbon chains but also odd and/or branched ones. To overcome such limitations, lipidomics researchers have contrived a novel procedure to quantify lipidomes comprehensively. Han and Gross (3) proposed a quantitative method for analyzing triglyceride molecular species using direct MS, which involves a procedure to calculate response factors for species that are not available as authentic materials, based on differences of the responses in the numbers of total carbons and double bonds among the species. For quantitative determination of phospholipid molecular species, Koivusalo et al. (4) utilized the following parame-

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Abbreviations: APCI, atmospheric pressure chemical ionization; CER, ceramide; CER[ADS], ceramide class consisting of α-hydroxy fatty acids and sphinganines; CER[AH], ceramide class consisting of α-hydroxy fatty acids and 6-hydroxy-4-sphingenine; CER[AP], ceramide class consisting of α-hydroxy fatty acids and 4-hydroxysphinganine; CER[AS], ceramide class consisting of α-hydroxy fatty acids and 4-sphingenines; CER[EOH], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 6-hydroxy-4-sphingenines; CER[EOP], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 4-hydroxysphinganine; CER[EOS], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 4-sphinnenines; CER[NDS], ceramide class consisting of non-hydroxy fatty acids and sphinganines; CER[NH], ceramide class consisting of non-hydroxy fatty acids and 6-hydroxy-4-sphingenines; CER[NP], ceramide class consisting of non-hydroxy fatty acids and 4-hydroxysphinganines; CER[NS], ceramide class consisting of non-hydroxy fatty acids and 4-sphingenines; ESI, electrospray ionization; LOD, limit of detection; LOQ, limit of quantification; NPLC, normal-phase liquid chromatography; RPLC, reversed-phase liquid chromatography; RSD, relative standard deviation; SC, stratum corneum; SIM, selected ion monitoring; S/N, signal to noise; TLC, thin-layer chromatography

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of two tables.

ters: (1) molar response factors among the different acyl chain lengths; (2) molar response factors among different degrees of unsaturation; and (3) molar response factors among the different head group classes.

In human cells and tissues, there are commonly three ceramide (CER) classes [combinations of nonhydroxy fatty acids and 4-sphingenines (sometimes referred to as sphingosines); nonhydroxy fatty acids and sphinganines (dihydrosphingosines); and α-hydroxy fatty acids and 4-sphingenines] that contain combinations of C_{18}-containing 4-sphingenines or sphinganines and even carbons-containing nonhydroxy or α-hydroxy fatty acids (13–15). CERs work as bioactive lipids relevant to signal transduction and cell regulation (16, 17). On the other hand, human stratum corneum (SC) is known to contain even more complex CERs which play important physicochemical roles in the barrier and water-holding functions of the skin as structural lipids (18, 19). Thus, it has been revealed that there are seven other CER classes [combinations of nonhydroxy fatty acids and 4-hydroxysphinganines (sometimes referred to as phytosphingosines); nonhydroxy fatty acids and 6-hydroxy-4-sphingenines; α-hydroxy fatty acids and 4-hydroxysphinganines; α-hydroxy fatty acids and 6-hydroxy-4-sphingenines; ester-linked ω-hydroxy fatty acids and 4-sphingenines; ester-linked ω-hydroxy fatty acids and 4-hydroxysphinganines; and ester-linked ω-hydroxy fatty acids and 6-hydroxy-4-sphingenines] in addition to the three common CER classes and the two covalently bound CER classes in the SC (20–23). However, the structures of such diverse CER species have not been completely characterized. Therefore, we performed structural analyses of those CER species in the SC using a normal-phase-LC-electrospray ionization-MS (NPLC-ESI-MS) technique, where 342 CER species were identified, a new CER class (the eleventh class, combinations of α-hydroxy fatty acid and sphinganines) was discovered, and many kinds of CERs with long chain-containing sphingoids were detected (24). Three-dimensional multi-mass chromatograms of the overall CER species in NPLC-ESI-MS were useful for CER profiling among different SC specimens but not for quantification (24). We further considered the necessity of a quantitative method for the overall CER species to study the statistical relationships between the CER lipidomes and the various types of SC and/or to clarify the de novo biosynthesis of long chain-containing CER species.

Regarding quantification of CERs in the SC, thin-layer chromatography (TLC) has been used conventionally (25–27). Although TLC can analyze levels of CER classes and remains a quite powerful tool, it cannot be applied to the quantification of individual CER species in the SC. There are other methods that can determine levels of individual CER species, such as HPLC-UV (28) and GC-MS (29) with a derivatizing technique, but the CER species targeted for quantification are restricted to only a few species. Over the last decade, many MS methods using electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) have been developed and applied to the analysis of CERs in human cells and tissues. Accompanied by such methodological advances, the technology to use MS has been employed for the analysis of CERs in human SC as follows: reversed-phase LC (RPLC)-ESI-MS (22, 30–32); ESI-MS (33, 34); RPLC-thermospray-MS (35); and NPLC-APCI-MS (36–39). However, those methods were not validated as quantitative methods for overall CER species in the SC, although some studies reported quantitative values based on unrealistic assumptions. Further, those methods do not have sufficient detection sensitivity to analyze CERs in smaller SC specimens.

The aim of this study was to develop a novel method for the comprehensive and highly sensitive quantification of CER species in the SC by improving the profiling method using a recently developed NPLC-ESI-MS technique (24). We successfully developed such a method using highly sensitive selected ion monitoring (SIM) measurement of as many as 182 kinds of molecular-related ions derived from overall CER species in NPLC-ESI-MS combined with a calculation procedure using relative responses of representative authentic CERs to quantify the overall CER species, including those not available as authentic materials. Herein, we demonstrate that this method can comprehensively quantify CER levels with high sensitivity and that it is useful for practical analyses.

MATERIALS AND METHODS

Nomenclature

CER classes are termed according to previous reports (21, 24, 40). Briefly, based on the terminology regarding constitutional fatty acids and sphingoids, the 11 CER classes are expressed as CER[NDS], CER[NS], CER[NP], CER[NH], CER[ADS], CER[AS], CER[AP], CER[AH], CER[EOS], CER[EOP] and CER[EOH]. For each species, the number of fatty acid carbons and unsaturation (if present) is expressed following the letters N, A, E, or O in parentheses while the number of sphingoid carbons is expressed following the letters DS, S, P, or H in parentheses. Also, total carbons of CERs and unsaturation of fatty acid (if present) are expressed following the letter C. For example, CER[EOS] with 68 carbons and 2 unsaturations in the fatty acid moiety is designated as $C_{68:2}$ CER[EOS].

Chemicals

Methanol, 2-propanol, chloroform and n-hexane, of HPLC grade from Kanto Reagents (Tokyo, Japan) were used to prepare SC lipid samples and mobile phases for HPLC. Ultra-pure water prepared using a Milli-Q purification system (Millipore, Bedford, MA) was used in all procedures. All other chemicals were of the highest analytical grade commercially available. Five authentic materials commercially available (purity > 98%), Ceramide $C_{12:0}$ (CER[N(12)S(18)]), Ceramide $C_{16:0}$ (CER[N(16)S(18)]), Ceramide $C_{17:0}$ (CER[N(17)S(18)]), Ceramide $C_{18:0}$ (CER[N(18)S(18)]), and Ceramide $C_{24:0}$ (Cer[N(24)S(18)]) were from Avanti Polar Lipids (Alabaster, AL). The following 18 authentic CERs were synthesized in our laboratory (24): CER[N(26)DS(20)], CER[N(10)S(20)], CER[N(22)S(20)], CER[N(26)S(16)], CER[N(22)S(24)], CER[N(26)S(20)], CER[N(30)S(18)], CER[N(26)S(24)], CER[N(30)S(20)], CER[N(26)P(20)], CER[N(26)H(20)], CER[A(26)DS(20)], CER[A(26)S(20)], CER[A(26)P(20)], CER[A(26)H(20)], CER[E(18:2)O(30)S(20)], CER[E(18:2)O(30)P(20)], and CER[E(18:2)O(30)H(20)] (> 95%).

Materials

The protocol was approved by the Ethical Committee of the Kao Corporation of Japan based on the Recommendations from the Declaration of Helsinki. Procedures for the collection of SC specimens with a tape-stripping method and for pretreatment of the SC-stripped tapes were performed with a slight modification of a previously reported method (24). Tape-stripped SC specimens were collected from three different regions each from the inner forearm and from the cheek of a healthy 35-year old Japanese male, who was enrolled in the study with informed consent. A polyphenylene sulfide film tape (Nichiban, Tokyo, Japan) with an area of 10 mm × 10 mm was pressed to the targeted skin and then stripped. The SC-stripped tape was then cut into two equal parts. One half of the tape was used to prepare extracted lipid samples. The tape stripped was immersed into methanol with sonication for 10 min. The lipid extracts were dried using a nitrogen stream and were then dissolved in chloroform/methanol (99.5:0.5, by vol). This lipid solution was applied to a Sep-Pak Vac RC silica cartridge (Waters, Milford, MA) that had been conditioned by chloroform/methanol (99.5:0.5, by vol), followed by solid phase extraction with 10 ml chloroform/methanol (99.5:0.5, by vol) and 10 ml chloroform/methanol (99:5, by vol). The latter fraction, which contains the CERs, was dried using a nitrogen stream and then dissolved in n-hexane/2-propanol/formic acid (95:5:0.1, by vol). An amount of lipid extracts from the tape was approximately 5 μg. The other was immersed into 0.1 M sodium hydroxide 1% (w/v) sodium dodecyl sulfate aqueous solution and was then incubated at 60°C for 2 h to obtain soluble proteins. After incubation, the solution was neutralized with 2 M hydrochloric acid aqueous solution.

Determination of soluble proteins

Concentrations of soluble proteins were determined according to the method of Smith et al. (41). A bicinchoninic acid protein assay kit (Pierce, Rockford, IL) and a microplate reader (Bio-Rad Laboratories, Hercules, CA) were used together with a calibration curve established using BSA.

Analysis using NP-HPLC-ESI-MS

The instrumentation was the same used for the comprehensive profiling of CERs in the SC (24). Thus, an Agilent 1100 Series LC/MSD SL (single quadrupole) system equipped with an ESI source, ChemStation software, an 1100 well plate autosampler (Agilent Technologies, Palo Alto, CA), and an Inertsil SIL 100A-3 1.5 mm i.d. × 150 mm column (GL Science, Tokyo, Japan) was used. In this LC/MSD system, two mobile phases (A, n-hexane/2-propanol/formic acid, 95:5:0.1, by vol; B, n-hexane/2-propanol/50 mM ammonium formate aqueous solution, 25:65:10, by vol) were used with a flow rate of 0.1 ml/min. The mobile phases were consecutively programmed as follows: a linear gradient of A (100–90%) and B (0–10%) between 0 and 3 min; a linear gradient of A (90–0%) and B (10–100%) between 3 and 35 min; an isocratic elution of A (0%) and B (100%) for 5 min; a linear gradient of A (0–100%) and B (100–0%) between 40 and 50 min; and an isocratic elution of A (100%) and B (0%) from 50 to 80 min for column equilibrium. The injection volume was 10 μl. The column temperature was maintained at 40°C. To enhance ionization of the molecular-related ions, 2.5 mM ammonium formate 2-propanol/methanol (50:50, by vol) at a flow rate of 0.1 ml/min was added to the mobile phase at the postcolumn position. ESI-MS parameters were as follows: polarity, positive; heater temperature of nitrogen gas, 300°C; flow of heated dry nitrogen gas, 8.0 l/min; nebulizer gas pressure, 20 psi; capillary voltage, 3500 V; fragmenter voltage, 150 V; cycle time; 1.15 s/cycle; dwell time for each ion, 7 ms. For SIM measurement, 182 kinds of molecular-related ions that have different m/z values and different retention times were simultaneously monitored by means of four monitoring channels and a time program designed in the mass spectrometer (see supplementary Table I). In quantifying levels of CERs in the three specimens each collected from the inner forearm and cheek SC of the subject tested, each crude extracted lipid sample was analyzed in triplicate. The CER levels in the inner forearm and the cheek were presented as the average values of the three specimens.

Evaluation of limit of detection (LOD), limit of quantification (LOQ), and linearity of calibration lines

Eleven authentic materials, CER[N(26)DS(20)], CER[N(26)S(20)], CER[N(26)P(20)], CER[N(26)H(20)], CER[A(26)DS(20)], CER[A(26)S(20)], CER[A(26)P(20)], CER[A(26)H(20)], CER[E(18:2)O(30)S(20)], CER[E(18:2)O(30)P(20)], and CER[E(18:2)O(30)H(20)], and an internal standard CER[N(17)S(18)] were used in these tests. The mixed authentic solution at a concentration of 4 nM each (40 fmol in 10 μl injection volume) was applied to the simultaneous SIM measurement in NPLC-ESI-MS, followed by the calculation of LOD and LOQ as signal to noise (S/N) being 3 and 10, respectively. Calibration curves were produced by injecting the mixed authentic solutions in a range between 4 and 128 nM (4, 8, 16, 32, 64, and 128 nM corresponding to 40, 80, 160, 320, 640, and 1280 fmol in 10 μl injection volumes, respectively). The concentration range for the calibration curves was based on preliminary experiments of the dynamic ranges.

Calculation of relative responses of authentic CERs

The mixed solutions including both the authentic materials over a concentration range between 10 and 100 nM (10, 20, 50, and 100 nM corresponding to 100, 200, 500, and 1000 fmol in 10 μl injection volumes, respectively) and the internal standard (CER[N(17)S(18)]) at a concentration of 50 nM (500 fmol in a 10 μl injection volume) were subjected to NPLC-ESI-MS and their calibration curves were then produced. Individual relative responses were calculated as ratios of slopes of calibration curves obtained from targeted authentic materials to that obtained from the internal standard.

Evaluation of inter-day and intra-day reproducibility

The 11 authentic materials belonging to different CER classes were used to clarify the influence of different hydrophilic structures on the relative responses. To evaluate the effect of different hydrophobic structures (chain lengths) within the same class on the relative responses, 12 authentic materials all belonging to CER[NS], CER[N(12)S(18)], CER[N(16)S(18)], CER[N(18)S(18)], CER[N(24)S(18)], CER[N(10)S(20)], CER[N(22)S(20)], CER[N(26)S(16)], CER[N(22)S(24)], CER[N(26)S(20)], CER[N(30)S(18)], CER[N(26)S(24)], and CER[N(30)S(20)], were used. Intra-day reproducibility was obtained from six consecutive analyses on the same day while inter-day reproducibility was obtained from six analyses on separate days over two months.

Calculation of recovery percentage

To estimate quantitative recovery percentage, the 11 authentic materials belonging to different CER classes and the internal standard were spiked in six replicates of crude lipid solutions extracted from the stripped SC to a final concentration of 50 nM each, followed by NPLC-ESI-MS analysis. Recovery percentage was calculated by comparing the determined levels of the 11 authentic CERs in spiked SC lipid samples with those in nonspiked SC lipid samples.

Determination of CER classes using TLC

TLC was performed according to the method of Imokawa et al. (42). Briefly, HPTLC-plates (silica gel 60 with concentrating zone, 20 cm × 10 cm; Merck, Darmstadt, Germany) were used for the separation. After aliquots of concentrated SC lipid samples collected from the inner forearm SC of two volunteers (samples A and B) were applied on a plate, it was developed twice with chloroform/methanol/acetic acid (190:9:1, by vol). After drying, the plates were sprayed with a 10% copper sulfate and 8% phosphoric acid solution and were then charred by heating at 180°C for 5 min. Each spot for CER classes was densitometrically determined using a TLC Scanner-3 photodensitometer (Camag, Muttenz, Switzerland) and a calibrated curve was established for each authentic material belonging to the same class as the targeted CERs. Their levels were compared with the total sum of the quantitative levels obtained by NPLC-ESI-MS.

RESULTS

Simultaneous SIM measurement in NPLC-ESI-MS

To detect CERs in the SC both with high sensitivity and with quantitative capability under the NPLC-ESI-MS conditions already optimized for the comprehensive profiling of CERs (24), we examined the practical availability of simultaneous SIM measurement in NPLC-ESI-MS. As it was theoretically impossible to separately detect each of the overall species as molecular-related ions in the SIM measurement due to the presence of many isobaric species, we decided to detect CERs consisting of isobaric species as apparent peaks in the SIM measurement. In the mass spectrometer used, four channels can be simultaneously monitored, in each of which the simultaneous monitoring for 30 kinds of ions is possible. Thus, 120 ions are usually maximal in simultaneous SIM measurements. However, we considered that more than 120 kinds of ions could be detected in one analytical run if the kinds of monitoring ions were changed depending on detection time, because each CER class has a different retention time under the conditions of this NPLC separation (24). As a result, we designed a time program using the four channels (see supplementary Table I), where as many as 182 kinds of molecular-related ions, including those derived from the overall species and the internal standard CER[N(17)S(18)], can be monitored.

To check that the NPLC-ESI-MS conditions are specific for CERs in SC, we examined the possibilities that SC lipids might ovelap with CERs. There are cholesterol, free fatty acids, cholesterol esters, and cholesterol sulfate within intercellular spaces in human SC (27, 43, 44), in addition to squalene, wax esters, and triglycerides that are excreted from sebaceous glands, and diglycerides, monoglycerides, and free fatty acids that are produced by their hydrolysis (45). Considering their molecular weights and polarities, it was readily judged that those lipids except for diacylglycerides do not overlap with the elution of any CERs under these NPLC conditions. As we could not eliminate a possibility that diacylglycerides might overlap with CERs, 10 kinds of authentic diacylglycerides were tested under these analytical conditions. As a result, all diacylglycerides tested eluted at 3 to 4.5 min under the NPLC conditions, as predominant molecular-ions $[M + NH_4]^+$ (data not shown), whereas the shortest retention time of CER species is 5.5 min (C54 CER[NDS]). This indicates that SC lipids, including diacylglycerides, do not interfere with the detection of CERs in the SC as long as these NPLC conditions are used.

Figure 1 shows representative multi-SIM chromatograms of CERs from the inner forearm and cheek SC under these NPLC-ESI-MS conditions. In this simultaneous SIM measurement for 182 kinds of ions, the S/N was about 30% of the SIM measurement for only one ion (data not shown), which indicates that the sensitivity in this simultaneous SIM measurement is lower than the ideal SIM measurement. Nevertheless, this method had more than a 10-fold higher sensitivity than the profiling method using scan measurement (24), as the simultaneous SIM measurement using only one piece of tape with an area 10 mm × 10 mm had almost the same S/N of peaks as the scan measurement using three pieces of tape with areas of 18 mm × 25 mm. At the very least, this indicates that the simultaneous SIM measurement of CERs in the SC is much more sensitive than our previous scan measurement method for comprehensive profiling.

To demonstrate the high sensitivity and quantitative performance of this method, LOD, LOQ, and calibration linearity for the 11 authentic different class CERs and the internal standard CER[N(17)S(18)] were examined. The LOD and LOQ for 10 μl injection volumes of the standard solution were in the range 3.8–9.8 and 13–32 fmol, respectively (**Table 1**). All correlation coefficients tested were greater than 0.9985 in the range 40–1280 fmol (Table 1), although there were slightly higher values of the intercepts, which may be ascribed to the asymmetrical shape and slight tailing of the peaks. Table 1 confirmed that the simultaneous SIM measurement has calibration linearity with a dynamic range in two figures and high sensitivity such that the LOD is at the fmol range. Based on these results, we determined that the internal standard should be added to each sample at a final concentration of 50 nM (500 fmol in 10 μl) so that its concentration corresponds to the center of the range of the calibration curves.

Procedure for estimating quantitative levels of overall CER species

We then examined the possibility of a procedure to estimate quantitative levels of overall CER species to comprehensively determine not only CERs available as authentic materials but also CERs that are not available. For this, relative responses were calculated for intra-day and for inter-day analyses. First, the 11 authentic different class CERs were used (**Table 2**). The responses of the 11 authentic CERs ranged from 0.43 for CER[A(26)P(20)] as the minimum to 1.67 for CER[N(26)DS(20)] as the maximum in the intra-day analyses when the internal standard CER[N(17)S(18)] was regarded as being 1.00. The responses were relatively reproducible in the intra-day analyses as seen in the range of relative standard deviation (RSD%) of 1.3–8.3%. As for the responses of inter-day

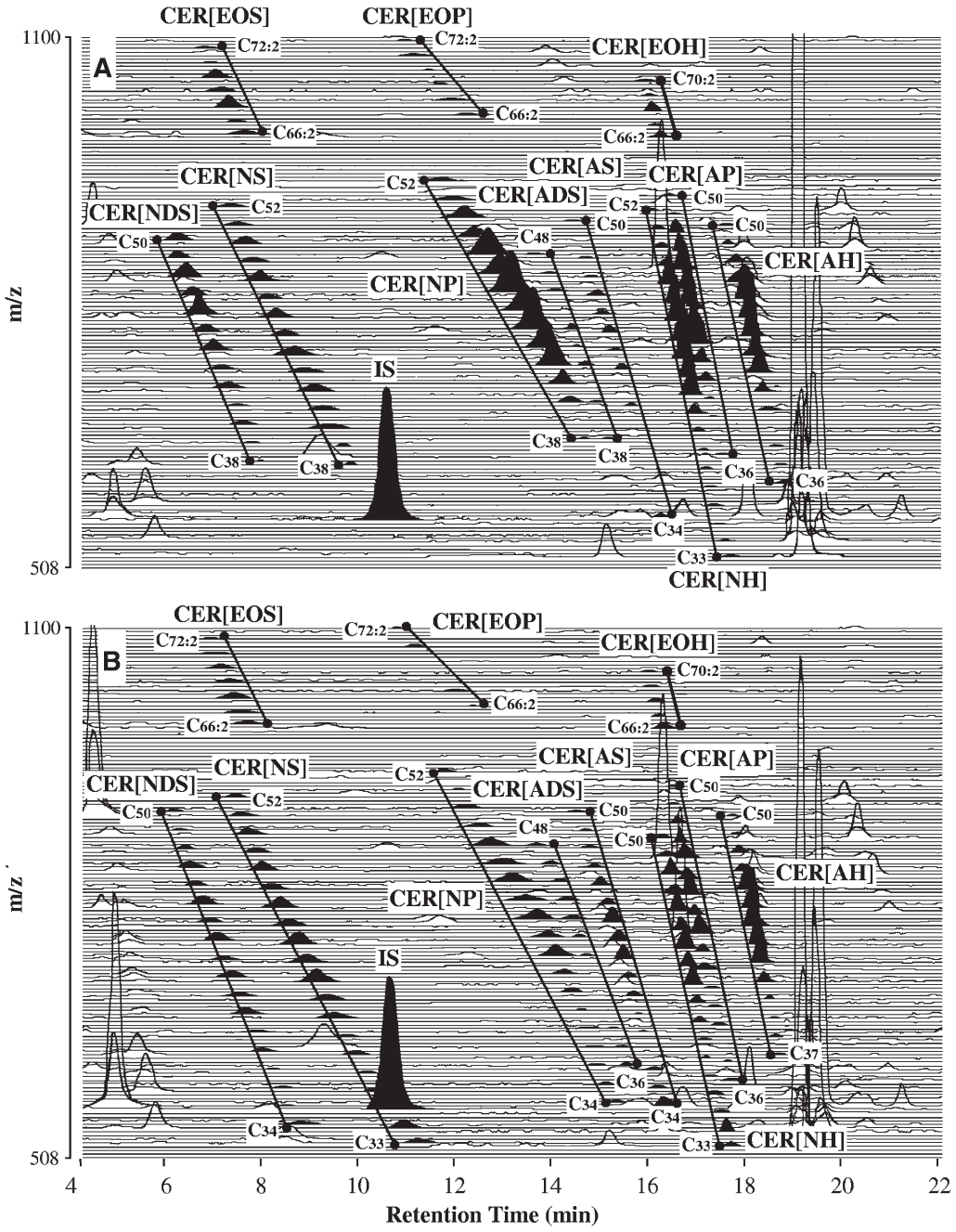

Fig. 1. Multi-SIM chromatograms of CER species in human SC using NPLC-ESI-MS. (a) inner forearm SC. (b) cheek SC. Sample: crude lipids obtained from a 35-year old male using a piece of tape 5 mm × 10 mm. NPLC conditions: column, Inertsil SIL 100A-3 (1.5 mm i.d. × 150 mm); column temperature, 40°C; mobile phase, (A) n-hexane/2-propanol/formic acid, 95:5:0.1, by vol, (B) n-hexane/2-propanol/50 mM ammonium formate aqueous solution, 25:65:10, by vol; flow rate, 0.1 ml/min; gradient elution, see Materials and Methods; injection volume, 10 μl; postcolumn addition, 2.5 mM ammonium formate containing 2-propanol/methanol (50:50, by vol) at a flow rate of 0.1 ml/min. ESI-MS conditions for simultaneous SIM measurement of 182 ions: polarity, positive; heater temperature of nitrogen gas, 300°C; flow of heated dry nitrogen gas, 8.0 l/min; nebulizer gas pressure, 20 psi; capillary voltage, 3500 V; fragmenter voltage, 150 V; cycle time; 1.15 s/cycle; dwell time for each ion; 7 ms.

analyses, CER[A(26)P(20)] had the minimal value 0.53 while CER[N(26)DS(20)] had the maximal value 1.58 with an RSD of 5.1–14.3%. Thus, the order of responses in the inter-day analyses was in close agreement with the order in the intra-day analyses, but the RSD tended to be slightly greater in the inter-day analyses. This indicates that the inter-day analyses seem to be slightly less reproducible than the intra-day analyses for different class CER species. Especially, the RSD of CER[N(26)H(20)], CER[E(18:2)O(30)S(20)], CER[E(18:2)O(30)P(20)], and CER[A(26)P(20)] in the inter-day were greater, as are 14.3%, 13.1%, 10.9%, and 10.7%, respectively (Table 2).

TABLE 1. LOD, LOQ, and calibration curves obtained under simultaneous SIM measurement conditions in NPLC-ESI-MS for 11 authentic different class CERs and an internal standard

	LOD[a]	LOQ[b]	Calibration curve[c]	
	fmol	*fmol*	*equation*	*R*
CER[N(26)DS(20)	7.1	23	y = 3833x + 23518	0.9998
CER[N(26)S(20)]	5.0	17	y = 3053x + 13003	0.9997
CER[N(26)P(20)]	6.1	20	y = 2313x – 15774	0.9996
CER[N(26)H(20)]	8.7	29	y = 1762x + 27403	0.9990
CER[A(26)DS(20)]	6.3	21	y = 2353x + 19581	0.9999
CER[A(26)S(20)]	9.8	32	y = 1480x + 10986	0.9999
CER[A(26)P(20)	4.9	16	y = 1230x + 9069	0.9998
CER[A(26)H(20)]	3.8	13	y = 1714x + 14760	0.9999
CER[E(18:2)O(30)S(20)]	6.8	22	y = 2339x – 7984	0.9999
CER[E(18:2)O(30)P(20)]	7.6	25	y = 1908x – 20743	0.9997
CER[E(18:2)O(30)H(20)]	4.3	14	y = 1392x – 11825	0.9999
CER[N(17)S(18)]	8.7	29	y = 2511x + 26579	0.9985

CER, ceramide; CER[ADS], ceramide class consisting of α-hydroxy fatty acids and sphinganines; CER[AH], ceramide class consisting of α-hydroxy fatty acids and 6-hydroxy-4-sphingenine; CER[AP], ceramide class consisting of α-hydroxy fatty acids and 4-hydroxysphinganine; CER[AS], ceramide class consisting of α-hydroxy fatty acids and 4-sphingenines; CER[EOH], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 6-hydroxy-4-sphingenines; CER[EOP], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 4-hydroxysphinganine; CER[EOS], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 4-sphinnenines; CER[NH], ceramide class consisting of non-hydroxy fatty acids and 6-hydroxy-4-sphingenines; CER[NDS], ceramide class consisting of non-hydroxy fatty acids and sphinganines; CER[NP], ceramide class consisting of non-hydroxy fatty acids and 4-hydroxysphinganines; CER[NS], ceramide class consisting of non-hydroxy fatty acids and 4-sphingenines; LOD, limit of detection; LOQ, limit of quantification; NPLC, normal-phase liquid chromatography; SIM, selected ion monitoring; S/N, signal to noise

[a] Defined as S/N = 3.

[b] Defined as S/N = 10.

[c] *R* is the correlation coefficient, x is the injected amount in fmol, and y is the peak area. All calibration curves were produced by injections of 40, 80, 160, 320, 640, and 1280 fmol.

However, there seemed to be no tendency that specific fatty acid or sphingoid moieties are related to the greater RSD. This might be related to relatively unstable performances of NPLC-ESI-MS, especially on separate days, compared with RPLC-ESI-MS that is stable and commonly used in the analyses of CERs (22, 30–32).

Next, 12 authentic CER[NS] with different carbons of fatty acid and sphingoid moieties were used (**Table 3**). Relative responses of the intra-day analyses were almost the same as those of the inter-day analyses except for CER[N(22)S(20)] showing slightly different values. Also, the RSD was similar to each other, as seen in the range of

TABLE 2. Relative responses of 11 authentic different class CERs

	Intra-day[a]		Inter-day[b]	
	Mean ± SD	*RSD %*	*Mean ± SD*	*RSD %*
CER[N(26)DS(20)]	1.67 ± 0.03	2.0	1.58 ± 0.09	5.7
CER[N(26)S(20)]	1.20 ± 0.01	1.0	1.21 ± 0.04	3.3
CER[N(26)P(20)]	0.82 ± 0.02	2.3	0.91 ± 0.05	5.9
CER[N(26)H(20)]	0.53 ± 0.03	4.8	0.65 ± 0.09	14.3
CER[A(26)DS(20)]	0.80 ± 0.07	8.3	0.97 ± 0.06	6.5
CER[A(26)S(20)]	0.54 ± 0.04	7.1	0.59 ± 0.03	5.4
CER[A(26)P(20)]	0.43 ± 0.01	3.4	0.53 ± 0.06	10.7
CER[A(26)H(20)]	0.62 ± 0.01	2.2	0.72 ± 0.06	7.8
CER[E(18:2)O(30)S(20)]	1.18 ± 0.06	5.3	0.96 ± 0.13	13.1
CER[E(18:2)O(30)P(20)]	0.81 ± 0.05	5.6	0.72 ± 0.08	10.9
CER[E(18:2)O(30)H(20)]	0.59 ± 0.01	1.3	0.57 ± 0.05	8.4

Relative response values are a ratio of the slope of the calibration curve (100–1000 fmol) each for 11 authentic CERs to that of the internal standard CER[N(17)S(18)].

CER, ceramide; CER[ADS], ceramide class consisting of α-hydroxy fatty acids and sphinganines; CER[AH], ceramide class consisting of α-hydroxy fatty acids and 6-hydroxy-4-sphingenine; CER[AP], ceramide class consisting of α-hydroxy fatty acids and 4-hydroxysphinganine; CER[AS], ceramide class consisting of α-hydroxy fatty acids and 4-sphingenines; CER[EOH], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 6-hydroxy-4-sphingenines; CER[EOP], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 4-hydroxysphinganine; CER[EOS], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 4-sphinnenines; CER[NH], ceramide class consisting of non-hydroxy fatty acids and 6-hydroxy-4-sphingenines; CER[NDS], ceramide class consisting of non-hydroxy fatty acids and sphinganines; CER[NP], ceramide class consisting of non-hydroxy fatty acids and 4-hydroxysphinganines; CER[NS], ceramide class consisting of non-hydroxy fatty acids and 4-sphingenines; RSD, relative standard deviation

[a] Six consecutive analyses on the same day.

[b] Six nonconsecutive analyses on different days over two months.

TABLE 3. Relative responses of 12 authentic CER[NS]

	Intra-day[a]		Inter-day[b]	
	Mean ± SD	*RSD %*	*Mean ± SD*	*RSD %*
CER[N(10)S(20)]	0.85 ± 0.01	1.5	0.84 ± 0.02	2.7
CER[N(12)S(18)]	0.94 ± 0.02	2.0	0.93 ± 0.01	1.4
CER[N(16)S(18)]	1.00 ± 0.02	1.7	1.01 ± 0.02	1.8
CER[N(18)S(18)]	1.03 ± 0.02	1.5	1.04 ± 0.01	1.2
CER[N(22)S(20)]	1.13 ± 0.01	1.3	1.27 ± 0.03	2.5
CER[N(24)S(18)]	1.25 ± 0.03	2.7	1.19 ± 0.02	1.5
CER[N(26)S(16)]	1.17 ± 0.01	1.1	1.12 ± 0.02	2.0
CER[N(22)S(24)]	1.35 ± 0.02	1.2	1.31 ± 0.02	1.8
CER[N(26)S(20)]	1.21 ± 0.01	1.0	1.19 ± 0.03	2.9
CER[N(30)S(18)]	1.27 ± 0.06	4.4	1.28 ± 0.05	3.9
CER[N(26)S(24)]	1.42 ± 0.02	1.6	1.36 ± 0.04	2.9
CER[N(30)S(20)]	1.28 ± 0.01	1.0	1.24 ± 0.03	2.7

Relative response values are the ratio of the slope of the calibration curve (100–1000 fmol) each for 12 authentic CERs to that of the internal standard CER[N(17)S(18)].

CER[NS], ceramide class consisting of non-hydroxy fatty acids and 4-sphingenines; RSD, relative standard deviation

[a] Six consecutive analyses on the same day.

[b] Six nonconsecutive analyses on different days over two months.

1.0–4.4% and 1.2–3.9% in the intra-day and inter-day analyses, respectively. This indicates that the intra-day and inter-day analyses may be reproducible for CER species belonging to the same class, compared with those belonging to different classes. As seen in Table 3, the responses seemed to increase with the increasing total carbons of the authentic CERs. Therefore we depicted the relationship between the total carbons and the responses for the 12 authentic CERs based on the inter-day result, where the regression curve is (relative response) = 0.0209 × (total carbon) + 0.2830 and the correlation coefficient (R) is 0.9320 (**Fig. 2**). There was no obvious tendency on which of carbons of fatty acids or sphingoids mainly determines the responses, because the order of the responses among isobaric species was unrelated to the numbers of their carbons. Although not shown in a figure, the intra-day relationship was similar (R = 0.9470). This was evidence indicating that the responses are positively correlated with the total carbons and implying that any relative responses of CER[NS] not available as an authentic material can be calculated if their total carbons are known.

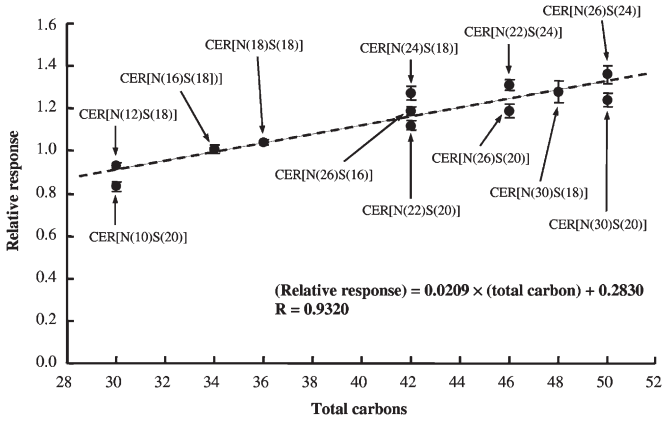

Fig. 2. Correlation between total carbons of 12 authentic CER[NS] and their relative responses in the inter-day analyses. Relative responses are presented as means ± SD. *R*, correlation coefficient.

Taken together with the above results, we designed a procedure for estimating quantitative levels of overall CERs in the SC using the simultaneous SIM measurement, based on the following assumptions: (1) relations between relative responses and total carbons in the linear regression observed in the 12 authentic CER[NS] may be fulfilled in others; (2) relations between relative responses and hydrophilic structures in the results on the 11 authentic different class CERs may be fulfilled in others; and (3) responses of isobaric species may be substantially similar to each other. In calculating levels of CERs according to those assumptions, we decided to use the responses obtained in the inter-day analyses since our analytical runs for the quantification would be done day by day. The level of a CER species (QL, ng/μg protein) can be calculated according to equation 1.

$$QL = 5 \times 10^{-5} \times RA \times MW / R_X / PL$$

where the constant 5×10^{-5} is the molar quantity of the added internal standard (nmol); RA represents the area ratio of the targeted CER to the internal standard; MW represents the molecular weight of the targeted CER; R_X represents the relative response of the targeted CER, which can be calculated according to equation 2; and PL represents the determined level of soluble proteins (μg).

$$R_X = R_{TC46} \times R_{NS} / 1.19$$

where R_{TC46} represents a relative response of an authentic CER belonging to the same class as the targeted CER but having 46 total carbons as shown in Table 2; R_{NS} represents the relative response of a CER[NS] with the same total carbons as the targeted CER, which can be calculated according to equation 3; and the constant 1.19 represents a relative response of CER[N(26)S(20)] in the inter-day analyses as shown in Table 3.

$$R_{NS} = 0.0209 \times TC + 0.2830$$

where the constants 0.0209 and 0.2830 represent slope and intercept, respectively, of the regression line shown in Fig. 2, and TC represents the number of total carbons of the targeted CER.

Recovery % of spiked authentic materials and comparison with the TLC method

We estimated the validity of comprehensive quantification using the simultaneous SIM measurement in NPLC-ESI-MS. First, recovery percentage was obtained by comparing the determined levels for the 11 authentic different class CERs in spiked crude SC lipids with those in the nonspiked crude lipids. The crude lipids were extracted from a 10 mm × 10 mm piece of a SC-stripped tape. **Table 4** shows the average and RSD of the recovery percentage, which are in the range of 81.8–94.9% and 2.1–12.5%, respectively. Although the RSD of CER[N(26)H(20)] and CER[A(26)P(20)] are somewhat higher, those are acceptable considering the practical analyses. This indicates that our method has acceptable accuracy and precision to determine the levels of the 11 CER species, even for the practical analysis in SC specimens of which some matrices coexist.

Quantitative levels of CERs determined in the SC using the newly developed method were compared with those using the conventional TLC method. Thus, crude SC lipid samples (samples A and B) were extracted from 10 consecutive SC-stripped tapes with areas of 15 mm × 15 mm from the inner forearm and were then applied to TLC analysis, followed by NPLC-ESI-MS analysis after the dilution of the lipid solutions. Since CER[NDS] and CER[ADS] are not separated from CER[NS] and CER[AS], respectively, on the TLC plate, those are presented as total sums in **Table 5**. In the method using NPLC-ESI-MS, the individual CER species were quantitatively determined both by the simultaneous SIM measurement and by the procedure for estimating the quantitative levels. The summed levels of the different CER classes are shown in Table 5. There were relatively greater differences in the levels between the TLC and NPLC-ESI-MS analyses, especially in the levels of the sum of CER[NDS] and CER[NS], and the sum of CER[ADS] and CER[AS] in sample A. Those differences were supposed to originate from vagueness of the TLC analyses for practical SC lipid samples. Thus, TLC spots of authentic CERs were definite due to application of only a single species in each spot, whereas the spots containing many CER species in the samples were so indefinite that it was difficult to distinguish from the neighbors' spots. However, statistical analyses using Student's *t*-test showed no significant differences between them ($P > 0.05$ in all pairs). This indicates that the levels estimated using this method are not substantially different from those obtained by the conventional TLC analytical method.

Application

In the multi-SIM chromatograms of CERs from forearm and cheek SC obtained from a 35-year-old male, we detected peaks of C_{38}-C_{50} CER[NDS], C_{38}-C_{52} CER[NS], C_{38}-C_{52} CER[NP], C_{33}-C_{52} CER[NH], C_{38}-C_{48} CER[ADS], C_{34}-C_{50} CER[AS], C_{36}-C_{50} CER[AP], C_{36}-C_{50} CER[AH], $C_{66:2}$-$C_{72:2}$ CER[EOS], $C_{66:2}$-$C_{72:2}$ CER[EOP] and $C_{66:2}$-$C_{70:2}$ CER[EOH] in the forearm SC, and those of C_{34}-C_{50} CER[NDS], C_{33}-C_{52} CER[NS], C_{34}-C_{52} CER[NP], C_{33}-C_{50} CER[NH], C_{36}-C_{48} CER[ADS], C_{34}-C_{50} CER[AS], C_{36}-C_{50} CER[AP], C_{37}-C_{50} CER[AH], $C_{66:2}$-$C_{72:2}$ CER[EOS], $C_{66:2}$-$C_{72:2}$ CER[EOP], and $C_{66:2}$-$C_{70:2}$ CER[EOH] in the cheek SC, as well as the internal standard CER[N(17)S(18)] (Fig. 1). Based on the comprehensive quantification for n = 3 each for the forearm and cheek SC specimens, total levels of the CERs were 27.8 ± 2.6 and 19.7 ± 1.3 ng/μg protein, respectively. Thus, in this subject, average CER levels from the three different regions were greater in the forearm SC than in the cheek SC. **Fig. 3** shows the average levels of different CER classes in the forearm and cheek SC. This indicates that the difference in average total levels between the forearm and cheek SC originates mainly from different levels of CER[NP], CER[AP] and CER[NH], although average levels of CER[NS] and CER[AS] were higher in the cheek SC than in the forearm SC. Further, in this subject, there were tendencies that average total carbons in CER[NDS], CER[NS], CER[NP], CER[NH], CER[AS] and CER[AH] of the forearm SC are greater than those of the cheek SC. A representative distribution of average total carbons in CER[NS] is depicted in **Fig. 4**. The forearm SC had higher levels of CER[NS] with larger total carbons while the cheek SC had higher levels of CER[NS] with smaller total carbons, in this subject. This demonstrates

TABLE 4. Recovery percentage of 11 authentic different class CERs spiked into crude extracted SC lipids

	Recovery percentage[a]	
	Mean ± SD	*RSD %*
CER[N(26)DS(20)]	84.7 ± 2.7	3.2
CER[N(26)S(20)]	94.9 ± 3.8	4.0
CER[N(26)P(20)]	85.7 ± 3.4	3.9
CER[N(26)H(20)]	89.3 ± 10.8	12.0
CER[A(26)DS(20)]	81.8 ± 6.4	7.8
CER[A(26)S(20)]	86.3 ± 5.3	6.2
CER[A(26)P(20)]	89.6 ± 11.2	12.5
CER[A(26)H(20)]	83.5 ± 5.3	6.3
CER[E(18:2)O(30)S(20)]	91.9 ± 2.1	2.3
CER[E(18:2)O(30)P(20)]	94.0 ± 2.0	2.1
CER[E(18:2)O(30)H(20)]	86.3 ± 7.0	8.1

CER, ceramide; CER[ADS], ceramide class consisting of α-hydroxy fatty acids and sphinganines; CER[AH], ceramide class consisting of α-hydroxy fatty acids and 6-hydroxy-4-sphingenine; CER[AP], ceramide class consisting of α-hydroxy fatty acids and 4-hydroxysphinganine; CER[AS], ceramide class consisting of α-hydroxy fatty acids and 4-sphingenines; CER[EOH], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 6-hydroxy-4-sphingenines; CER[EOP], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 4-hydroxysphinganine; CER[EOS], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 4-sphinnenines; CER[NH], ceramide class consisting of non-hydroxy fatty acids and 6-hydroxy-4-sphingenines; CER[NDS], ceramide class consisting of non-hydroxy fatty acids and sphinganines; CER[NP], ceramide class consisting of non-hydroxy fatty acids and 4-hydroxysphinganines; CER[NS], ceramide class consisting of non-hydroxy fatty acids and 4-sphingenines; RSD, relative standard deviation; SC, stratum corneum

[a] Six replicates.

TABLE 5. CER levels[a] in the inner forearm SC using the conventional TLC method and the newly developed NPLC-ESI-MS method

	Sample A[b]		Sample B[b]	
	TLC	NPLC-ESI-MS	TLC	NPLC-ESI-MS
CER[NDS]		3.02 ± 0.05		1.58 ± 0.02
CER[NS]	4.53 ± 0.98[c]	2.76 ± 0.02	3.16 ± 0.66[c]	2.03 ± 0.08
CER[NP]	13.0 ± 1.81	11.5 ± 0.34	5.77 ± 0.65	5.16 ± 0.29
CER[NH]	7.94 ± 1.77	9.47 ± 0.17	7.63 ± 1.54	7.47 ± 0.19
CER[ADS]		0.38 ± 0.03		0.24 ± 0.05
CER[AS]	2.35 ± 0.80[d]	1.15 ± 0.07	1.80 ± 0.45[d]	1.43 ± 0.03
CER[AP]	5.46 ± 0.67	5.90 ± 0.16	5.44 ± 0.51	5.96 ± 0.19
CER[AH]	5.01 ± 0.53	5.49 ± 0.12	5.24 ± 0.92	6.02 ± 0.15
CER[EOS]	1.57 ± 0.16	1.79 ± 0.02	1.62 ± 0.24	1.42 ± 0.04
CER[EOP]	0.80 ± 0.22	0.62 ± 0.02	0.13 ± 0.04	0.09 ± 0.02
CER[EOH]	1.70 ± 0.33	1.34 ± 0.05	0.87 ± 0.24	0.61 ± 0.02
Total	42.39 ± 6.13	43.45 ± 0.30	31.66 ± 4.72	32.01 ± 0.55

CER, ceramide; CER[ADS], ceramide class consisting of α-hydroxy fatty acids and sphinganines; CER[AH], ceramide class consisting of α-hydroxy fatty acids and 6-hydroxy-4-sphingenine; CER[AP], ceramide class consisting of α-hydroxy fatty acids and 4-hydroxysphinganine; CER[AS], ceramide class consisting of α-hydroxy fatty acids and 4-sphingenines; CER[EOH], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 6-hydroxy-4-sphingenines; CER[EOP], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 4-hydroxysphinganine; CER[EOS], ceramide class consisting of ester-linked non-hydroxy fatty acids, ω-hydroxy fatty acids and 4-sphinnenines; CER[NH], ceramide class consisting of non-hydroxy fatty acids and 6-hydroxy-4-sphingenines; CER[NDS], ceramide class consisting of non-hydroxy fatty acids and sphinganines; CER[NP], ceramide class consisting of non-hydroxy fatty acids and 4-hydroxysphinganines; CER[NS], ceramide class consisting of non-hydroxy fatty acids and 4-sphingenines; NPLC, normal-phase liquid chromatography; RSD, relative standard deviation; SC, stratum corneum; TLC, thin-layer chromatography

[a] Mean ± SD (ng/μg protein).

[b] Collected from the inner forearm SC of two volunteers. Three replicates each in TLC and NPLC-ESI-MS analyses.

[c] Presented as the total sum of CER[NDS] and CER[NS].

[d] Presented as the total sum of CER[ADS] and CER[AS].

that the method is highly sensitive for quantifying overall CER species in the SC, as they can be determined in only one SC-stripped tape piece as small as 5 mm × 10 mm.

DISCUSSION

This study was aimed at developing a method for quantifying overall CER species in the SC with high sensitivity based on an NPLC-ESI-MS technique already constructed for the comprehensive profiling of CERs using scan measurement (24). As a result, by designing a new time program for SIM fit to the chromatographic elution of 11 different CER classes, we could optimize novel conditions for simultaneously measuring as many as 182 kinds of molecular-related ions derived from the diverse CER species in the SC. The results on the 11 authentic different-class CERs indicate that this method is useful for highly sensitive quantification. Thus, the calibration curves are linear over the dynamic range of at least two figures, and the LOD is in the fmol range, even during the simultaneous measurement of as many as 182 kinds of ions. Therefore, the use of SIM measurements to achieve highly sensitive quantification has been very successful. As exemplified in this study, the method is so highly sensitive that we can analyze diverse CER species in only one SC-stripped tape piece as small as 5 mm × 10 mm. Further, using this method, the authentic CERs are quantitatively recovered as seen in the recovery test in which the authentic CERs were spiked in the crude extracts of SC lipids, indicating that there are no ion suppression effects that interfere with the quantitative detection of the authentic CERs.

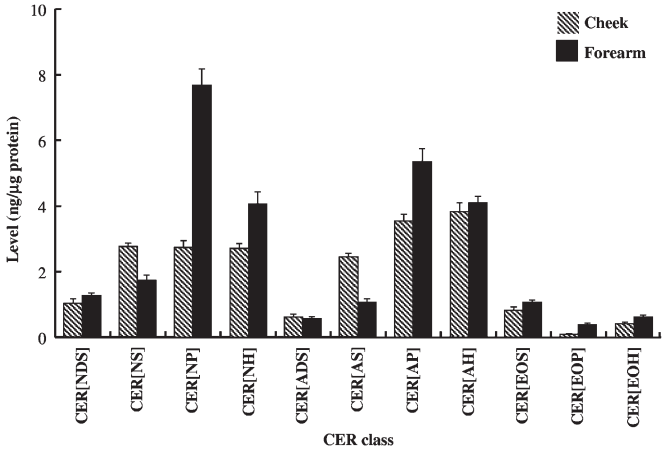

Fig. 3. Average levels of CER classes in three different regions each of the forearm and cheek SC of a 35-year-old male. Levels are presented as means ± SD.

To comprehensively quantify diverse CER species in the SC, it would be ideal to get all authentic materials for individual CER species that exist in the SC and to produce their calibration curves in every analytical run together with the internal standard. However, such an analytical run would be realistically impossible. Referring to previous studies on triglycerides (3) and phospholipids (4), we used another strategy: (1) obtain a systematic rule for relative responses of CER species to the internal standard by using authentic CERs limited to but covering all CER

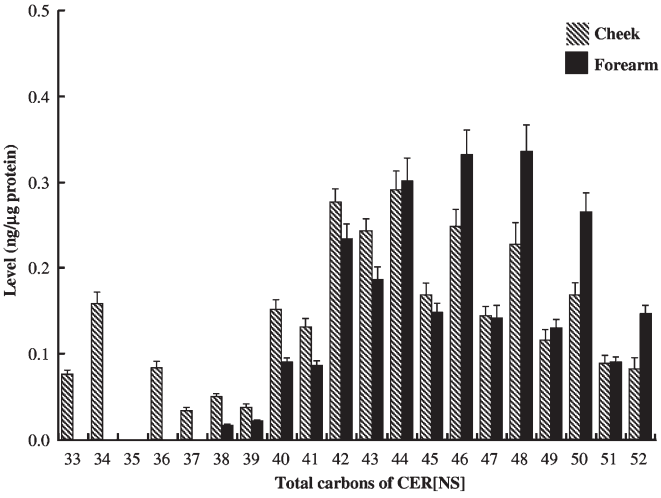

Fig. 4. Average levels of CER[NS] species in three different regions each of the forearm and cheek SC of a 35-year-old male. Levels are presented as means ± SD.

species present in the SC and (2) theoretically estimate quantitative levels of CER species not available as authentic materials according to the systematic rule. To derive such a rule, we considered that at least two types of authentic CERs would be required: one type including CER standards belonging to 11 different classes but having identical total carbons, and the other including CER standards belonging to the identical class but having different total carbons. The first type was determined to be 8 authentic CERs having 46 total carbons that consist of 26 and 20 carbons in the fatty acid and sphingoid moieties, respectively, for N- or A-containing CERs, and 3 authentic CERs having 68 total carbons consisting of 18, 30, and 20 carbons in E, O and sphingoid moieties, respectively, for EO-containing CERs, because those are commonly present in the SC (24). As for the second type, we prepared 12 authentic CERs belonging to CER[NS] with 30–50 total carbons. To evaluate differences in relative responses among isobaric species, the combination of constitutional fatty acids and sphingoids was also taken into consideration. Using the responses of those authentic CERs, a proper procedure for estimating the quantitative levels was developed.

One must bear in mind a total systematic error originating from this estimation when using it routinely as a comprehensive quantification. According to equations 1, 2, and 3, when an error in measuring soluble proteins is ignored, the systematic error comes from the four following factors: (1) the area ratio of the targeted CER species to the internal standard; (2) the response of an authentic CER species belonging to the same class as the targeted species but having 46 total carbons; (3) the slope and intercept in the regression curve produced by different CER[NS] species; and (4) the response of CER[N(26)S(20)]. In addition, we have to consider a fifth factor: the responses of different isobaric species. In the inter-day analyses in a sequential run, the average RSD of area ratios for the 11 authentic standards, 1.4% (data not shown) can be used for factor 1. The average RSD of the responses for the 11 authentic standards, 8.4% (Table 2) can be also used for factor 2. Regarding factor 3, the average RSD of the slopes and intercepts in six regression curves, which were produced on six separate days (Table 3), can be available. This error value was calculated as $3.7\% = (2.9\%^2 + 2.3\%^2)^{1/2}$, where the values 2.9% and 2.3% represent the RSD of the slopes and intercepts, respectively. The error of factor 4 directly corresponds to the RSD of the response for CER[N(26)S(20)], 2.9% (Table 4). Since these error values directly reflect the realistic variations, we judged that they would be adequate to use. On the other hand, we thought that the error value corresponding to factor 5 could be estimated using average residual differences between the measured and superimposed responses of four isobaric sets within the class CER[NS] (two species of C30 CER[NS], three species of C42 CER[NS], two species of C46 CER[NS], and two species of C50 CER[NS]). The average difference of these nine species was 4.5%. Although these error values are rough estimates and not completely precise, they are based on actual data. So we judged that they would be sufficient to roughly estimate the total systematic error. According to the above considerations, the total systematic error could be theoretically estimated to be $\pm 21.4\%$ $[2\sigma = 2 \times (1.4^2 + 8.4^2 + 3.7^2 + 2.9^2 + 4.5^2)^{1/2}]$ of the levels determined in the inter-day analyses. This error value seems relatively large. However, levels of CERs can be discriminated if their differences are approximately 20% in the inter-day analyses, and such a difference occurs frequently in biological analyses. In our application using only a single subject, we can clearly mention that, at least in this subject, there are distinct differences in the total levels and the levels of CER[NS], CER[NP], CER[NH], CER[AS], CER[AP], CER[EOP] and CER[EOH] between the forearm and cheek SC, because the levels differ from each other by 20%. However, we must wait for further analyses using multiple subjects to make it clear whether the differences in CER classes/species compositions between distinct SC regions are common in humans.

Although the accuracy of this method was evaluated for the 11 authentic CERs by spiking them into crude SC lipids, we could not directly validate the quantitative recovery percentage for other CERs that are not available as authentic CERs but are present in the SC. Further, we could not validate the apparent quantitative levels for apparent peaks consisting of different isobaric species. However, we currently consider that quantitative levels estimated for nonvalidated CERs may roughly have similar accuracy to those for the authentic CERs. That's because the summed quantitative levels of CERs using the NPLC-ESI-MS technique were comparable to those determined by TLC, even though our method involves the theoretical procedure already described. Strictly speaking, those levels do not completely coincide, but such differences in levels may come from great differences in the analytical principles/procedures between NPLC-ESI-MS and TLC, especially from vagueness of the TLC analyses in practical SC lipid samples. Therefore, the conventional method using TLC that has been traditionally used to quantify the CER class levels in the SC can be replaced by this newer NPLC-ESI-MS

method that has advantages for highly sensitive quantification of the overall CER species.

Herein we compare this novel method with previously reported methods for quantification of CERs. As already noted, the LOQ for the 11 authentic CERs was 13–32 fmol using the simultaneous SIM measurement. Of all previous reports describing methods for the quantification of CERs, the methods reported by Han (15) and by Masukawa and Tsujimura (46) are highly sensitive. In Han's method (15), which uses direct MS and neutral loss ion scan, the LOD was approximately 10 fmol (consumed) by examination using an authentic CER[NS] commercially available. Provided that the LOD and LOQ are determined according to the S/N of 3 and 10, respectively, our method would have almost the same LOQ as that method. The other method (46), which uses RPLC-ESI-MS, has an LOQ of 0.48–1.37 fmol for 13 authentic CER[NDS], CER[NS], CER[ADS] and CER[AS] that exist in human hair, which indicates higher sensitivity of that method than our method. However, it should be noted that in both those methods, the targeted species are restricted to 2 or 4 classes. At least our method covering as many as 11 classes has the highest sensitivity for CER species in the SC. On the other hand, regarding the comprehensive performance, our method should be compared with methods reported by Farwanah et al. (36–39) and by Muñoz-Garcia et al. (47). Farwanah et al. (36–39) developed a NPLC-APCI-MS method for analyzing CER species in the SC, which is superior to other earlier methods in terms of profiling performance and has been used for clarifying differences in CER species between healthy and unhealthy human skin (36–38). However, the values derived from that method are not guaranteed as quantitative levels, so they eventually used both the levels of the CER class derived from TLC and the relative abundances of the CER species obtained from NPLC-APCI-MS for the comparison between healthy and unhealthy SC (37). This indicates that the method reported by Farwanah et al. (36–39) is not very useful for the quantification of overall CER species in the SC. In another method which used RPLC-atmospheric pressure photospray ionization-MS to analyze CER species in sparrow SC, reported by Muñoz-Garcia et al. (47), each CER species belonging to CER[NS], CER[AH], CER[EOS] and CER[EOH] was quantified using an internal standard CER[N(17)S(18)] based on the assumption that the internal standard may have the same molar response as each of the CER species targeted. Although the total level of CERs obtained by that method agreed well with those determined by the TLC method, this agreement does not guarantee validity of the method for the quantification of individual CER species. The authors actually noted in their paper that "until further verification, our quantification results remain inconclusive" (47). From the viewpoints of high sensitivity and quantitative performance, we can conclude that our novel method is superior to the other previous methods for analyzing CER species in the SC.

Finally, we describe perspectives using the newly developed method. In this study, we developed a new tool to detect subtle differences and changes which might be closely related to CER functions in SC. It seems that the species with longer chains in fatty acid and/or sphingoid moieties are critically important for the understanding of the CER functions. The structures of those species were already identified using NPLC-ESI-MS/MS (24). Based on this quantitative method, we can know their levels. However, when extremely higher or lower levels of the species with longer chains are observed in some SC samples, we should identify their structures in the samples using NPLC-ESI-MS/MS again because it might lead to new findings on CER functions in the SC. As this method has extremely high sensitivity, we can quantify the overall species in only one SC-stripped tape piece as small as 5 mm × 10 mm, which allows an almost noninvasive analysis of CER species in human SC without any distress for the subjects tested. This implies that atopic dry skin and facial skin could be analyzed with this method, specimens of which are usually too painful to collect with organic solvent extractions (37) or cyano acrylate-stripping (42). This method enabling quantitative lipidomics approaches will be helpful for future studies clarifying the physicochemical and physiological roles of CER species in the SC, including advances in dermatological and cosmetic science.

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