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Optimization of Ceramide Analysis Method Using LC-MS in Cosmetics

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Abstract : Ceramide is a lipid in which sphingoid bases and fatty acids are linked by amide bonds. As a marker of skin disease in the human stratum corneum, its disease-causing and therapeutic effects have been partially confirmed, and it is therefore an important element in commercially available cosmetic formulations. However, structural diversity caused by differences in the chain length, number, and location of hydroxyl groups makes quality control difficult. In this study, a method was established to separate different ceramide species using reversed-phase LC-MS/MS and thus enable qualitative evaluation. Separation of four standards was achieved within a short retention time, and the accuracy and sensitivity of the method were demonstrated by the low limit of detection (LOD) calculated based on the calibration curve showing linearity, with $R^2 > 0.994$. After verification of reproducibility and reliability through intra- and inter-day analyses, the efficiency of the method was confirmed through analysis of commercial cosmetic raw materials.

Keywords : Ceramide, Cosmetics, LC-MS/MS

Introduction

Ceramides are substances that make up various parts of the human body and are distributed from nerves to skin. The stratum corneum of the skin contains the greatest amounts and diversity of ceramides, which affect the maintenance of skin health. In diseases such as psoriasis and atopy, quantitative differences in ceramide have been reported between affected and unaffected areas. To resolve this issue, ceramide substances have been applied to problem areas to study their effects on recovery.[1–2] Although their efficacy has not been confirmed, there is a great deal of interest in products containing ceramides, many of which are available commercially for cosmetic applications. These products contain a variety of ingredients, including ceramide NP to improve the skin barrier.[3] Therefore, it is important to verify the accuracy of the listed ingredients and content information for product reliability and quality assurance. However, previous analytical studies on raw materials have focused on other additives and impurities, and there have been few studies designed for the identification of ingredients to confirm the reliability of commercial products. Ceramides are a subclass of sphingolipids in which sphingoid bases (SBs) and fatty acids are linked by amide bonds. Humans have five types of SB, also called long-chain base (LCB): S (sphingosine), P (phytosphingosine), DS (dihydrosphingosine), H (6-hydroxysphingosine), and SD (4,14-sphingadienne). These are combined with four types of N-acyl fatty acids: N (non-hydroxy), A (α-hydroxy), O (ω-hydroxy), and EO (esterified ω-hydroxy). Many ceramide types can be produced depending on the number of carbons in each chain, as well as simple combinations of the two chains. Therefore, research is continuing to determine the types and distributions of ceramides in various biological samples.[4–6]

Liquid chromatography-mass spectrometry (LC-MS) is widely used in ceramide analysis as it is useful for separating different types of ceramides and for obtaining information on their compositions.[7–9] However, optimization of the

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conditions for the analysis of lipids, including ceramides, is important due to the diversity of analysis conditions according to the mobile phase solvents and additives used.[10]

In this study, optimization of the method for separation and analysis of ceramide types in the stratum corneum was performed by ultraperformance liquid chromatography (UPLC), using a reversed-phase column and quadrupole time-of-flight mass spectrometer (Q-TOF) to obtain high-resolution structural information. The method was validated by evaluating the limit of detection (LOD), linearity, reproducibility, and precision. The raw materials contained in the ceramide mixture were analyzed using the Folch lipid extraction method, and our method was then applied.[11]

Materials and methods

Materials

Ceramide NS (d18:1/18:0), NP (t18:0/18:0), AP (t18:0/24:0), and EOS (d18:1/30:0/18:2) were purchased from Sigma-Aldrich (St. Louis, MO, USA), Avanti Polar Lipids, Inc. (Alabaster, AL, USA), and Cayman Chemical (Ann Arbor, MI, USA).

LC-MS-grade water and acetonitrile (ACN), LC-grade methanol (MeOH), isopropyl alcohol (IPA), HPLC-grade chloroform ($CHCl_3$), and reagent-grade ammonium formate were all obtained from Sigma-Aldrich. LC-MS-grade formic acid was purchased from TCI Chemicals (Tokyo, Japan). Cosmetic raw materials used as samples were provided by MacroCare Inc. (Cheongju, South Korea).

Sample preparation

Standards were prepared and used at a concentration of 10 µg/mL. A calibration curve was plotted using final concentrations of 10 ng/mL, 50 ng/mL, 100 ng/mL, 500 g/mL, 1 µg/mL, and 2.5 µg/mL. Commercial raw materials are manufactured with various additives, including surfactants and preservatives, to improve their ease of use. Therefore, ceramide was extracted using a modified lipid extraction method based on the Folch method.[11] First, 5 µL of the sample was added to 1 mL of $CHCl_3$/MeOH/water (2:1:1,v/v). After vortex mixing for 5 min, the sample solution was centrifuged at 7,500 rpm for 10 min. The supernatant was removed from the separated phase and gently dried under a stream of nitrogen gas. The dried lipid extract was resuspended in $CHCl_3$/MeOH (4:1, v/v) and diluted with IPA.

LC-MS/MS conditions

Ceramide analysis was performed using a Waters SELECT SERIES Cyclic IMS cyclic (Waters Corporation, Milford, MA, USA) coupled to an ACQUITY UPLC I-CLASS PLUS system. In UPLC, separation was performed using an ACQUITY UPLC CSH C18 column (inner diameter 2.1 mm; length 100 mm; particle size 1.7 µm; Waters). Mobile phase A consisted of ACN:water (3:2, v/v), and mobile phase B consisted of IPA:ACN (9:1, v/v), both containing 10 mM ammonium formate. For the mobile phase B, 0.1% formic acid was added. The flow rate was 0.3 mL/min, and the injection volume was 5 µL. The gradient of the mobile phase was 0–17 min, 40%–95%B; 17–19 min, 95%B; 19.01–20 min, 40%B. Ceramide standards and raw material extracts were analyzed in positive ion mode using an electrospray ionization source. Other MS parameters were as follows: capillary voltage, 2.5 kV; cone voltage, 40 V; source temperature, 140°C; desolvation temperature, 600°C. Analyses were performed in the *m/z* range of 50–1,200, and the collision energy was 20–60 eV.

Results and discussion

Optimizing the LC-MS/MS method for ceramide analysis

As SBs with 18 carbon atoms are frequently used in commercially available cosmetic products, standards with the corresponding carbon numbers were used. The results of MS/MS analysis of each standard material confirmed a peak corresponding to the $[M+H]^+$ ion in the spectra, and fragments of the N-acyl fatty acid chain of [P] and [S] were also identified. Therefore, a mixture of standards made at a concentration of 10 µg/mL was used to optimize the method based on the confirmed mass spectra. A previous study investigating the effectiveness of additives in lipid analysis showed that the addition of 10 mM ammonium formate resulted in the greatest improvement in ionization and signal intensity in positive ion mode.10 Therefore, mobile phases consisting of mixtures of ACN and MeOH with IPA were compared to establish optimal analytical conditions with the addition of formic acid (Table 1).

Table 1. Methods used for ceramide analysis

Meth.	Additives	Mobile phase A				Mobile phase B			
		Solvent	v/v	Solvent	v/v	Solvent	v/v	Solvent	v/v
a	10mM AF*+ 0.2 % FA	Water	100	-	-	MeOH	100	-	-
b	10mM AF+ 0.2 % FA	Water	100	-	-	ACN	95	Water	5
c	10mM AF	Water	10	MeOH	90	MeOH	50	IPA	50
d	10mM AF	Water	40	ACN	60	ACN	50	IPA	50
e	10mM AF	Water	40	ACN	60	ACN	10	IPA	90
f	10mM AF + 0.1 % FA	Water	40	ACN	60	ACN	10	IPA	90

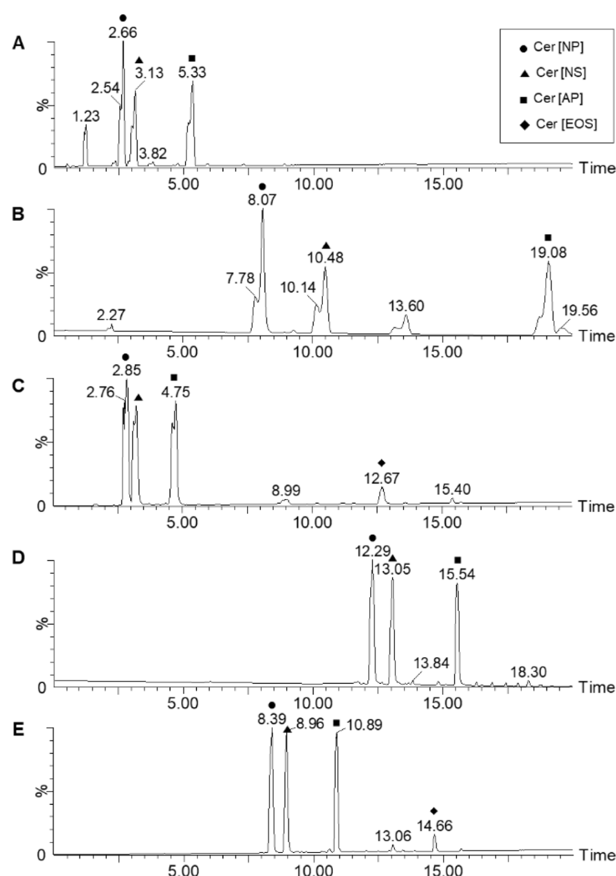

Figure 1. BPI (Base peak intensity) Chromatograms of ceramide standards applying solvent composition and additives changes. A through E is listed in order from methods a to e.

In the case where a single mobile phase consisting of ACN and methanol was used (Meth. a, b), the initial gradient was changed to 95%B, and the flow rate was changed to 0.5 mL/min under the LC conditions described above (Fig. 1A, B). However, only three standards appeared under both conditions, with peak splitting occurring due to the initial mobile phase ratio.[12] ACN showed peak broadening due to its lower backpressure than methanol but showed better separation between standard peaks. For elution of all standards, mixing with IPA to increase solvent strength was attempted (Meth. c, d) (Fig. 1C, D). Four standards appeared only with the 5:5 (v/v) mixture of methanol and IPA. This was thought to be caused by a decrease in retention time due to high backpressure. However, peak splitting still occurred, and the initial peaks within the retention time were not well separated. In the case of ACN, selectivity increased due to improved resolution, and peak broadening was improved. Therefore, Method e (Fig. 1E), which was based on Method d, improved the IPA ratio for elution of the four standards. All four standards eluted within the set retention times. In addition, the addition of formic acid improved all peak intensities by 1.2 times while maintaining the same chromatogram pattern as in Method e. Therefore, Method f was selected as the optimal method for the present study.

The LOD was used to evaluate the sensitivity of the optimized method. Standards prepared at concentrations of 10 ng/mL, 50 ng/mL, 100 ng/mL, 500 ng/mL, 1 μg/mL, and 2.5 μg/mL were used, and analysis was repeated three times per concentration. The relation between the concentration of each standard and the extracted ion chromatogram (XIC) peak area of $[M+H]^+$ ions was determined using linear regression analyses. From the obtained calibration curve, the LOD was determined according to the formula LOD = 3.3 × SD/S, where SD represents the standard deviation of the XIC peak area, and S represents the slope of the calibration curve. As a result, the LOD showed low values between 60 and 90 ng/mL for the four standards, indicating that the sensitivity of the analysis method was sufficient.

The R^2 value was > 0.994, indicating linearity. The reproducibility of the method was verified by intra- and inter-day analyses. Five samples were prepared and measured over 3 consecutive days using a standard concentration of 1 μg/mL. The intra- and inter-day variations of the analysis were determined as the relative standard deviation (RSD), calculated using the XIC peak area of the $[M+H]^+$ ion of the standard. RSD (%) was calculated using the formula (SD/average XIC area) × 100. The intra-day RSD (%) showed a value within a maximum of 4.43%, and the inter-day RSD (%) also showed a value within 2%-10%. Therefore, these results indicated the reliability of the optimized method based on reproducibility and precision (Table 2).

Cosmetic sample analysis

The chromatogram of the raw sample analyzed after pretreatment showed peaks consistent with the retention time of the standard (Fig. 2A). For the RT 8.39 min peak, the retention time not only matched that of NP (t18:0/18:0) but also the product ion. The *m/z* value of 584 corresponding to

Table 2. Method validation parameters of an LC-MS/MS method optimized for ceramide analysis

No.	Type	R^2	LOD (mg/mL)	Intra-day (n = 5) (RSD (%))	Inter-day (n = 5) (RSD (%))
1	Cer [NS]	0.994	0.090	1.14	2.43
2	Cer [NP]	0.998	0.060	2.65	9.92
3	Cer [AP]	0.999	0.059	2.20	4.44
4	Cer [EOS]	0.998	0.079	4.43	4.42

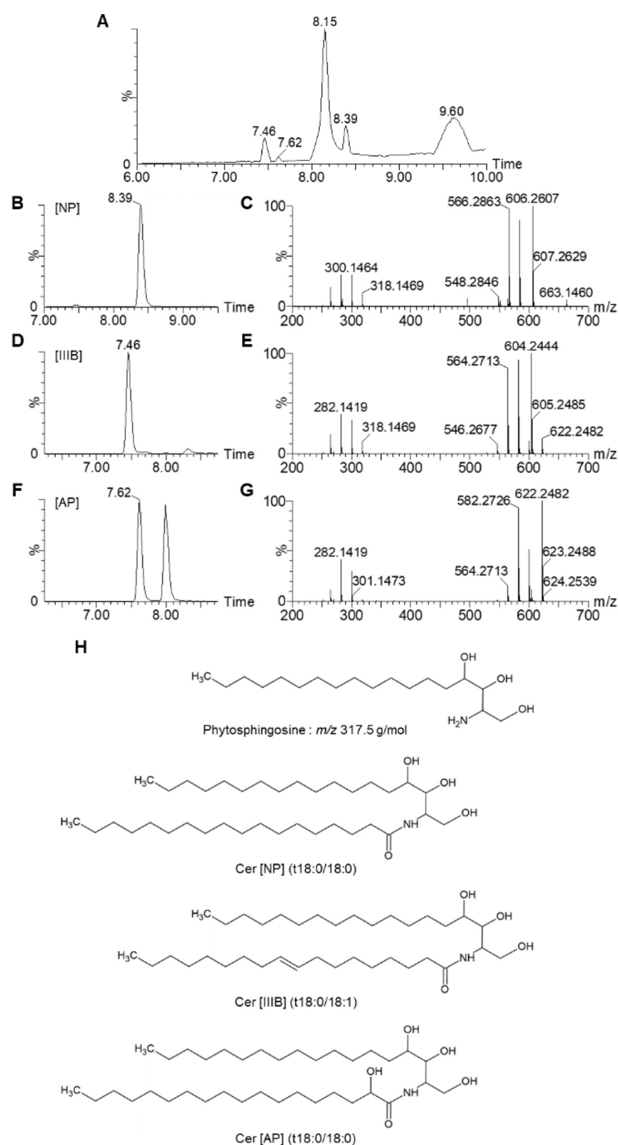

Figure 2. Chromatogram and mass spectrum of cosmetic raw material sample extract. A is the enlarged BPI chromatogram, and the XIC and MS/MS spectra of Cer [NP], [IIIB], and [AP] identified in the samples correspond to B–C, D–E, and F–G, respectively. H represents the structure of phytosphingosine and each ceramide.

$[M+H]^+$ of NP, and the *m/z* values of 318, 300, 282, 264 of phytosphingosine corresponding to $[M+H]^+$, $[M+H-H_2O]^+$, $[M+H-2H_2O]^+$, $[M+H-3H_2O]^+$, were confirmed (Fig. 2B, C, H). The product ions at the peaks of RT 7.46 min and 7.62 min also had *m/z* values of 318, 300, 282, and 264, indicating that their structures contained phytosphingosine. In the case of $[M+H]^+$, *m/z* 582 was 2 Da smaller, and *m/z* 600 was 16 Da larger than NP (Fig. 2D-G). Therefore, the front peak was IIIB (t18:0/18:1), which has one double bond in the N-acyl chain, and the rear peak was assumed to be AP (t18:0/18:0) with OH present in the N-acyl chain. In Fig. 2F, the two peaks of XIC indicate the presence of stereoisomers along the OH direction. Other peaks were identified as impurities.

Conclusions

With advances in research and technology for skin care and disease treatment, the use of ceramides in commercial products, such as cosmetics, is increasing. In this study, a method for extracting ceramide from commercially available raw materials and conducting qualitative analysis was developed based on the Folch method, a representative technique for lipid extraction. The optimized LC-MS/MS method was both rapid and highly reliable based on its sensitivity and selectivity. In the future, developments based on this method could extend its application to samples with matrices containing various ceramides.

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