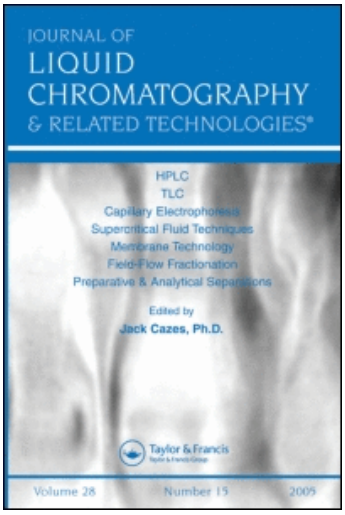

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Quantitative Analysis of Ceramide III of *Saccharomyces cerevisiae* by Normal Phase HPLC

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ABSTRACT

Ceramide III was prepared by the cultivation of *Saccharomyces cerevisiae*. Ceramide III was partitioned from the cell extracts by solvent extraction and analyzed by normal phased high performance liquid chromatography (NP-HPLC) using evaporative light scattering detector (ELSD) and mass spectrometer. The mobile phase was composed of chloroform and methanol. One linear gradient mode was applied to gain proper analysis of cell extracts. Quantitative analysis of ceramide was performed. Based on the analytical condition, the effect of cultivation temperature for the production of ceramide was investigated and the optimum cultivation temperature was found to be 30°C at pH 7. From the results of mass spectrometer analysis, C26 ceramide was the dominant cell culture. The fragment ions from the mass spectrum of

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ceramides III, which were of various molecular weights, from $m/z = 237$–677, were observed.

Key Words: Ceramide III; Temperature; pH; Quantitation.

INTRODUCTION

The intercellular lipid of stratum corneum protects the organism against undesirable influences from the environment and the compositions are ceramide 40%, cholesterol 25%, fatty acid 25%, and cholesterol solvate 10%. Among them, ceramide is known as one of the most important sphingolipids that play significant roles as structural and functional components in biomolecular membranes.[1] Ceramides are an important class of sphingolipids, which have an equally significant role as structural and functional component in biomolecules membranes.[1] Ceramides are lipids of major importance involved in the barrier function of the skin.[2] Ceramides consist of a fatty acid bound to the amino group of sphingoid base or another related long chain base.[3] Ceramides also constitute the hydrophobic backbones of the complex sphingolipids: sphingomyelin, cerebrosides, gangliosides, etc.[4]

Today's issue of world cosmetic industries is the production of ceramide from vegetable sources instead of animal sources that have problems like bovine spongiform encephalopathy. The studies of sphingolipid production from yeast are advanced nowadays.[5] Compared to other materials, yeast is a relatively suitable source for production of ceramide because it has advantages such as fast growth, non-toxic,[1] and easy mutation.[6] In spite of this advantage, quantitative study of ceramide production from yeast is not clear and most quantity of production depends on only two strains *Saccharomyces cerevisiae*[1] and *Torulopsis (Candida) utilis*.[6]

The classical approach in ceramide analysis is based on thin layer chromatography (TLC).[7–9] However, this method has several disadvantages, such as quantitative separation of individual species being very difficult[2] and incorrect. If a better specific analysis is demanded, liquid chromatography or gas chromatography are the methods of choice. Since ceramides do not show a sufficient volatility, the polar hydroxy groups have to be subjected to derivatization,[9] specifically in gas chromatography. In recent years, high performance liquid chromatography (HPLC) has been applied to analyze ceramides.[9–13] Non-aqueous reverse phased (NARP) HPLC systems was determined to be suitable for analysis of highly hydrophobic backbones of ceramides.[10,12] As ceramides are classified as their polar hydroxy groups and long chain based fatty acids, normal phased high performance liquid chromatography (NP-HPLC) was determined to be the analysis tool for the

polar hydroxy group. Ceramides revealed the weak chromatographic functionality in the range of 200–210 nm region of UV detector.[10] More recently, evaporative light scattering detector (ELSD) has been reported as the appropriate detection method for analysis of ceramides.[2,12]

The purpose of this work is to find the optimum condition of cell cultivation for ceramide from *S. cerevisiae* in terms of the amounts of ceramides using NP-HPLC with ELSD. We cultivated *S. cerevisiae* with different temperature conditions with pH conditions. The binary mobile phase of chloroform and methanol were used as mobile phases.

EXPERIMENTAL

Chemicals

The HPLC grade solvent, chloroform, IPA, and methanol were from J. T. Baker (Philipsburg, NJ). Ceramide III mixtures standard were purchased from Sigma (St. Louis, MO).

Instrumentation and Method

Cell Cultivation

Yeast Strain and Culture Condition

The strain used in this research was *S. cerevisiae* (KCCM 50515), which is known as a sphingolipid producer. The cultivation medium was YEPD (glucose, 20 g/L; bacto peptone, 20 g/L; yeast extract, 10 g/L).[6] Cultivation was performed in Erlenmeyer flasks on the rotary shaker.[9] Cultivation condition was 200 rpm and the cultivation time was 48 hours.

Recovery of Lipid

Centrifugation and ultrasonification methods were used for recovery of lipids. *S. cerevisiae* were harvested in the stationary growth phase by centrifugation at 4000 rpm and washed twice with distilled water.[14]

Extraction of Lipid

Five grams (wet weight) of yeast cells were suspended in 20 mL of a chloroform–methanol mixture (1 : 2, v/v), and the mixture was sonicated three times in a sonicator (IKA U200S) for 5 min. The disrupted cell suspension was filtered through 0.2 μm RC filter (Sartorius). The residual cell debris was

suspended in 50 mL of chloroform–methanol mixture (2 : 1, v/v) and stirred by magnetic stirrer for 30 min at room temperature and filtered again. This process was repeated four times.[9] Production of ceramide was examined at temperature changes of 20°C, 25°C, 30°C, 35°C, and 40°C and with pH change at 4, 5, 6, 7, and 8. The extraction method was the same as the above process.

Preparation for High Performance Liquid Chromatography

The cell extracts were 150 mL before any pretreatments. The cell extracts were concentrated into 3 mL with a rotary evaporator. The concentrations were adjusted with chloroform. As the extracts contained small amount of water, the phase separation occurred after concentration with chloroform. Only the chloroform phase was selected with a pipette.

Normal Phase High Performance Liquid Chromatography

High performance liquid chromatography was performed with a Waters 600S solvent delivery system (Waters, Milford, MA). The data acquisition system was Autochro 2.0 installed on a HP Vectra 500 PC. Chromatograms were stored in the RAW format. The detection system used was ELSD from Alltech (Deerfield). The drift temperature was set as 75°C and the flow rate of the nebulizer gas (nitrogen) was 1.6 L/min.

The mobile phases were degassed with helium. The flow rate of the mobile phase was fixed at 1.0 mL/min. The mobile phases were chloroform and methanol for NP-HPLC, respectively. The one step gradient condition used was chloroform/methanol = 90/10–30/70, gradient time 3 min. The column used in this work was a generous gift from RStech (Daejeon, Korea). The dimension of the column was 250×4.6 mm. The packing material was OP Sil-51002546 (Daejeon, Korea). The injection amount was fixed as 5 μL. This experimental was carried out in ambient temperature.

LC/MS

QUATTRO LC Triple Quadrupole Tandem Mass Spectrometer (Micromass, UK) equipped with an electrospray interface (ESI) was used throughout. Electrospray ionization was performed, preferably, in the negative ion full scan mode. Tuning parameters was ES+. The voltage of entrance was 22.0 and exit was 17.0 V, respectively. Direct insert probe (DIP) means that the sample

is directly injected into the mass spectrometer by syringe pump, without passing through the column. Because the eluent from the column contained large amount of chloroform, the DIP method was applied to analyze the fractioned ceramide III sample. Ion energy was set as 0.8 V. The nebulizer gas flow rate was 84 L/hr.

RESULTS AND DISCUSSION

Quantification of Ceramide III with Evaporative Light Scattering Detector

The calibration curve of ceramide III was estimated to confirm the amount of ceramide in cell extracts. Ceramide III was injected ranging from 0.01 to 0.05 mg. The response of ELSD for ceramide with NP-HPLC was nonlinear as shown in Fig. 1. However, the calibration curve was fitted as a linear correlation. The calibration curve equation was $y = 4.218 \times 10^{-8}x$, where y is the amount (μg) of injected ceramide and x is area (mV sec). The value of regression coefficient was 0.966. The one linear gradient elution (chloroform/methanol = 90/10–30/70 vol.%, 3 min) was employed throughout to obtain the proper analysis time and resolutions.

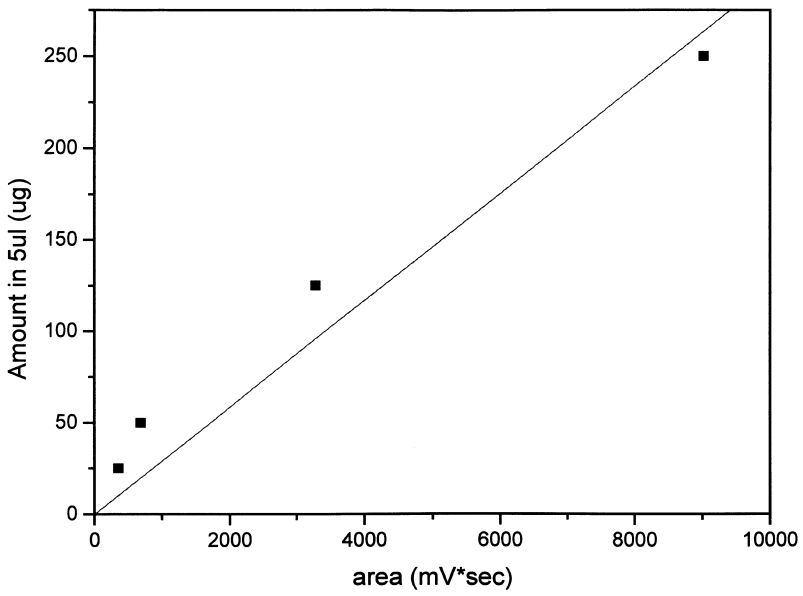

Figure 1. Calibration curve of ceramide III for normal phased HPLC with ELSD.

Optimum cell cultivation conditions producing ceramide III from *S. cerevisiae* were studied, according to temperature and pH using NP-HPLC. As mentioned above in the introduction, since ceramide was characterized with its polar hydroxyl groups as I, II, III, IX, etc., and long-chain sphingoid base and fatty acid, normal phased like silica gel was the choice of the analysis tool because of polarity dependence adsorption. RP-HPLC could be chosen for the determination of long-chain sphingoid base and fatty acid. The optimum cell cultivation condition was found in terms of the contained amount of ceramide in the cultivated cell extracts. Ceramide III was identified by comparing the standard retention time. The amounts of ceramide III by *S. cerevisiae* from the cultivation were shown Table 1. From Table 1, the largest amount of ceramide III was observed at 30 and pH 7. The exact amount of ceramide III from the first cultivation was 32.27 μg/5 μL. At 35°C, results were barely influenced by the pH conditions as shown in Table 1. The extreme conditions of cultivations, such as 40°C or pH 8, showed smaller amounts of ceramide III than others conditions. The optimum condition was reasonable because Rupecic and Maric pointed that *S. cerevisiae* showed best activation at 30°C and pH 7 (1).

Direct Insert Probe Analysis

Ceramide III was fractionated with the best condition of *S. cerevisiae*, 30°C and pH 7, by NP-HPLC. The chromatogram of 30°C and pH 7 was shown in Fig. 2. The fraction time was 3.2 to 3.8 min. Because the mobile phase eluted from the column contained large amounts of chloroform, which was very volatile, the fractioned sample and standard mixture of ceramide III could not be detected with on-line, but could be detected with the DIP method. The mass spectrums of standard mixtures of ceramide III and fractioned samples were shown in Figs. 3 and 4, respectively. The various ranges of

Table 1. The amount of ceramide III (μg/μL) by *S. cerevisiae* according to temperature and pH conditions.

	pH				
Temperature	4	5	6	7	8
20	0	3.49	0	1.06	4.41
25	4.01	5.18	0	4.96	5.68
30	4.63	4.20	5.91	6.45	3.26
35	3.98	3.41	3.20	3.31	2.89
40	1.65	1.29	3.34	4.09	0.24

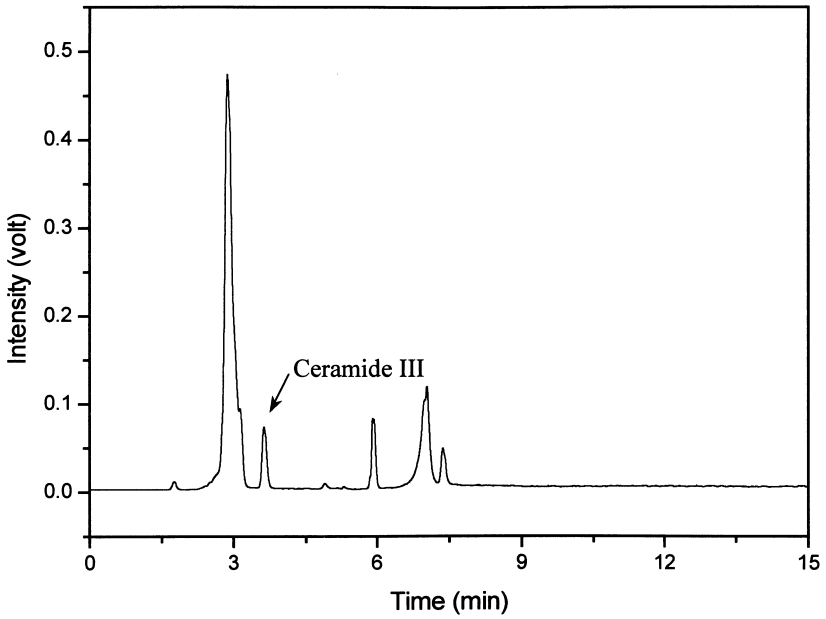

Figure 2. Chromatogram of cultivated cell extract at 30°C and pH 7 by NP-HPLC with ELSD.

molecular mass were identified from the analysis of the DIP of both of the standard mixtures of ceramide III and fractionated samples. In the case of the standard mixture of ceramide III, the fragmented ions range was 212 to 685 m/z. The largest peak has been detected at $m/z = 549$ in the standard mixture of ceramide III from Sigma. However, the fractionated sample revealed the largest peak at $m/z = 342$ and 580. However, $m/z = 342$, did not correspond with the retained standard ceramide III mixture. The peak at $m/z = 398$ in Fig. 4 was characterized for stearic acid moiety in the ceramide.[9] The relative abundance for both of ceramide III standard mixture and fractionated ceramide III were listed in Table 2. From Table 2, the most abundant component from fractionated ceramide III from *S. cerevisiae* was $m/z = 580$, which was revealed as C26.[1]

In summary, the optimum condition producing ceramide III for cultivation of *S. cerevisiae* was found using NP-HPLC with ELSD. The optimum condition of cultivation was 30°C and pH 7. On the basis of the presented data, it might be concluded that yeast ceramides displayed quite heterogeneous

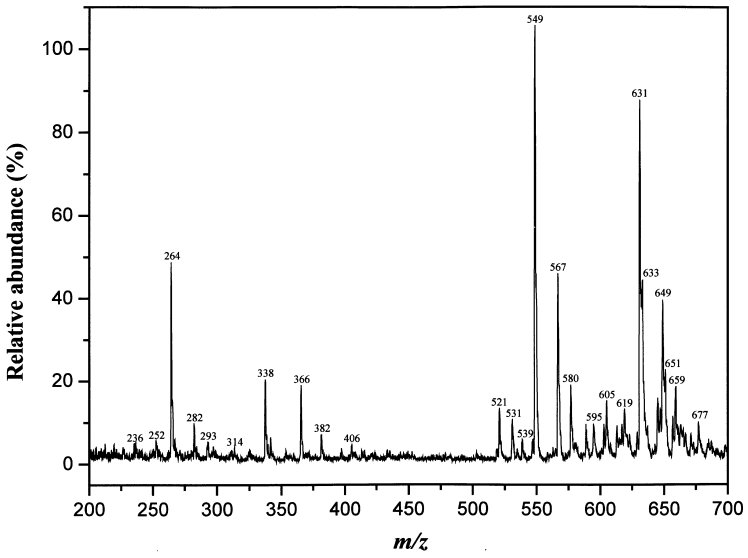

Figure 3. Mass spectrum of ceramide III standard mixture with DIP method.

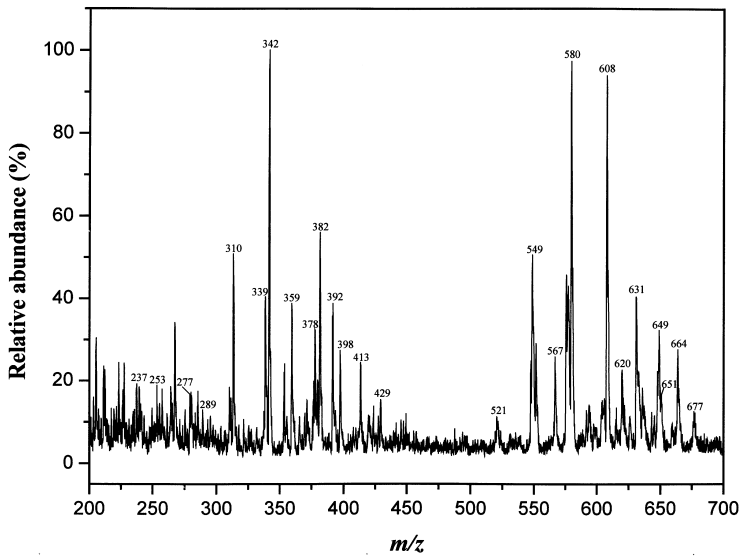

Figure 4. Mass spectrum of fractioned ceramide III by *S. cerevisiae* with DIP method.

Table 2. Relative abundance of ceramide III based on the most abundant component.

Standard mixture of ceramide III		Fractioned sample	
Molecular mass	Relative abundance (%)	Molecular mass	Relative abundance (%)
236	1.8	237	19.4
252	5.5	253	19.0
264	48.8	—	—
282	9.9	279	16.9
293	5.6	—	—
314	2.4	313	50.9
338	7.2	338	8.9
366	5.5	342	93.2
382	3.5	382	56.1
406	2.7	—	—
521	12.8	521	10.2
531	9.5	—	—
549	100.0	549	50.7
567	41.9	567	22.7
580	16.9	580	91.1
595	8.4	—	—
619	13.3	620	20.2
631	87.8	631	40.0
649	39.6	649	27.1
659	18.7	—	—
677	10.2	677	10.8

structure, depending on the yeast strain and growth conditions. The highest portion of sphingoid base and fatty acids was C26 from DIP spectrum analysis.

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