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Quantitation of yeast ceramides using high-performance liquid chromatography–evaporative light-scattering detection

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Abstract

A high-performance liquid chromatograph equipped with an evaporative light scattering detector (ELSD) (HPLC–ELSD) was used to assay the ceramides in yeast cells. The HPLC–ELSD method employed a cyanopropyl bonded column (CN column) that effectively separated the main interfering substance ergosterol without any derivatization process; most other interfering substances were also removed. The method can be applied for routine assay of ceramide content in yeast.

Keywords: Yeast cells; Ceramides

1. Introduction

Sphingolipids are a very complex lipid class. They are found in all eukaryotic cell membranes, serving as structural and functional components [1–3]. As structural components, they improve the rigidity of cell membranes [4,5], acting as anchor attachment sites for glycosylphosphatidylinositol (GPI)-anchored proteins [6]. As signal molecules, sphingolipid metabolites are involved in many important biological processes, such as signal transduction, growth regulation and stress responses [7]. Among the complex sphingolipids, ceramides, the key component, are of special interest, because they are the backbone of all sphingolipids. Ceramides play important roles in all the processes. Many studies focused on the role of ceramides in different cells under various circumstances [8–11].

The growing interest in ceramides requires accurate quantitative analytical methods. So far, the most recognized methods include radioactive labeling of ceramide precursors and subsequent extraction and separation by thin layer chromatography (TLC) [12–14]; HPLC separation followed by derivatization and HPLC separation combined with mass spectrometry (MS) detection [15–20]. The radiolabelling methods were widely used for ceramide identification and quantitation for many years; most conclusions on the biological roles of ceramides were obtained using these methods. However, care should be taken with the selection of radioactive precursor as the precursors might join other metabolic pathways. In addition, the radioactive labels were generally expensive and hazardous to handle. To avoid utilizing radiolabels, Iwamori developed a method for the derivatization of ceramides with benzoyl chloride or

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benzoyl anhydride, which brought ceramides an ultraviolet (UV) absorptive tag at 230–280 nm [16]. This approach allowed for the quantitative determination of ceramide contents in various biological samples. Despite the good quantitative results, the Iwamori procedure is time-consuming and cumbersome, the key reaction is very sensitive to water, and all reagents have to be prepared fresh each time and handled anhydrously. The derivatives of ceramides are unstable, requiring immediate handling. Previati reported a method for determining ceramide concentration via coupling a fluorescent label [17]. The reaction was achieved after prolonged incubation at −20 °C. The authors reported an 80% yield after 3 h of incubation. The linearity was in a range of 5 to 500 ng. However, the oxydril group of phospholipids could inhibit the reaction, thus some extra purification steps before the labeling reaction must be applied to remove the interfering lipids and optimize the reaction conditions. Yano et al. developed a method for quantitative analysis of molecular species of ceramides by reversed-phase (RP) chromatography [18]. Ceramide species were separated according to the chain length of fatty acids and long chain base backbone. The method is useful for elucidating the functions of particular subspecies of ceramides. However, long incubation time is required for successful derivatization, bringing a drawback to this method. Mass spectrometry (MS) is a powerful tool for its high level of sensitivity and selectivity. It could successfully determine the ceramide level and molecular species, however, MS is not a routine analytical instrument due to its expensive running costs. A simple and reliable method was not available until the introduction of the evaporative light-scattering detector. McNabb used the detector to analyze the ceramides extracted from yeast without any previous purification or derivatization [21]. In McNabb's method, peaks of the ceramides were very close to that of the interferences, which could easily cover the ceramide peaks, making the quantitation difficult. In addition, the silica column employed in the methods was also vulnerable to contamination from other complex lipids in cell extracts.

In this paper, we describe a method for the separation of cell lipid contaminants from ceramides in yeast cell extracts. The peaks were clearly resolved with each other, making the quantitation work possible without any derivatization and purification. This study presents the possibility to analyze ceramide content without expensive chemicals and difficult handling.

2. Materials and methods

2.1. Materials

Four kinds of ceramides were analyzed. Ceramide 4 (*N*-palmitoyl-phytosphingosine) and ceramide 5 (hydroxy-palmitoyl-phytosphingosine) were gifts from Cosmoferm (The Netherlands). Ergosterol, triglycerides, 1,2-diacyl-sn-glycerol-3-phosphocholine (PC), sphingomyelin, ceramide III and ceramide IV were purchased from Sigma (USA). Fig. 1 illustrates the structures of different ceramides and ergosterol.

Hexane, ethanol and chloroform were purchased from Dikma (Beijing, China), all were HPLC grades. Other reagents used were at least analytical grade.

2.2. Cell culture

Saccharomyces cerevisiae strains 1912, 4608 and 1408 were used for general studies. Yeast *Candida lipolytica* was kindly provided by Dr. J. Rupcic (Department of Chemistry and Biochemistry, Medical Faculty, University of Rijeka, Croatia). This strain was reported to produce ceramides at relatively high level (up to 0.5% of dry cell weight under proper conditions) [22]. Cells were first cultivated in inoculation medium (glucose 2%, peptone 0.6%, malt extract 0.3%, yeast extract 0.3%) and then in production medium supplemented with 1% glucose as carbon source instead of 2% in the inoculation medium. Batch cultivation was performed on a shaker at 200 rev./min and 30 °C (NBS, BIOFLO3000, USA). Culture (200 ml) was harvested in mid-exponential phase at 4000 *g* and washed twice with distilled water. The cells were suspended in 32 ml chloroform–methanol (1:2), broken under a sonicator (Xinzhi, NingBo, China) (400 W, 0.9 s operation, 0.6 s interval, lasting for 30 min). Broken cells were then extracted by the

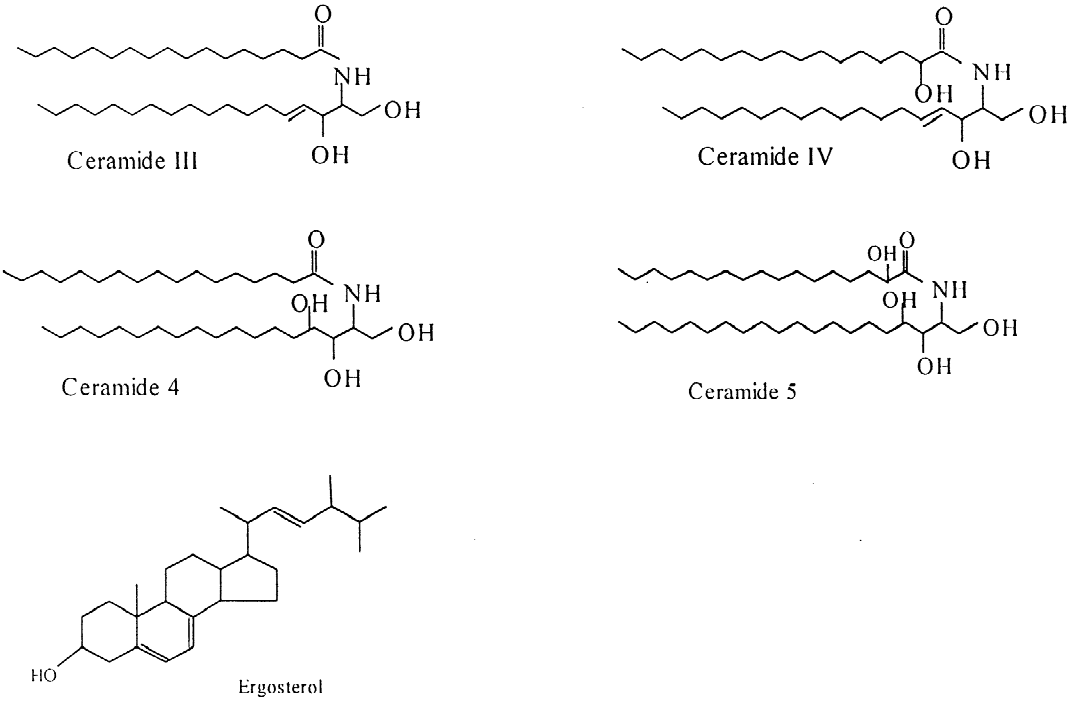


Fig. 1. Structures of ceramide III, ceramide IV (from Sigma), ceramide 4, ceramide 5 (from Cosmoferm) and ergosterol. The carbon chain length on the FA side of ceramide III and ceramide IV varied from 16 to 24. The ceramide 5 is a mixture of two isomerization forms.

method of Bligh and Dyer [23], washed with water to remove most polar lipids and non-lipid contaminants. The lipid extracts were then subjected to mild alkaline hydrolysis by adding one quarter of 1 *M* methanolic NaOH, staying at 55 °C for 30 min, to remove most glycerol lipids. The mixture was extracted by adding 0.8 volume of water and 2 volumes of chloroform to remove the water-soluble products. The organic phase was then evaporated under vacuum and redissolved in a proper amount of chloroform for HPLC assay.

2.3. High-performance liquid chromatography

The HPLC system consisted of a Thermo Separation Pump P2000, equipped with a vacuum degasser and a 20-μl manual injector (Thermo Separation, USA). The data were collected by JiangSheng workstation software (Dalian, China) and then processed by Origin (Microcal, USA). Two HPLC systems were employed in the separation: the first consisted of a 5-μm silica column (4.6×250 mm) (Hypersil, UK), a guard column of the same material (4.6×3.0 mm) (Phenomenex, USA) and the mobile phase consisted of chloroform and ethanol, running under isocratic or gradient modes. The mobile phase for the isocratic mode was chloroform–ethanol (90:10), flow-rate was 0.6 ml/min. The gradient mode started with pure chloroform for 1 min, followed by a 25-min linear gradient between chloroform and chloroform–ethanol (75:25), the column was then re-equilibrated for 10 min (flow-rate 1.0 ml/min). The second HPLC system consisted of a 5-μm CN column (4.6×150 mm) (Alltech, USA), the mobile phase was hexane–ethanol (99:1) (flow-rate 1.0 ml/min). The column was washed with ethanol for 90 min to remove the polar lipids after every 30 injections. A Sedex 75 ELSD (Sedere, Alfortville, France) was employed, with the temperature of the drift tube set at 40 °C and gas pressure at 3.5 bar, the gain is usually set at 8 or 6.

3. Results and discussion

3.1. The interference of ergosterol in the analysis of ceramides on the HPLC–ELSD system

There have been many reports on the analysis of ceramides; in most cases, pure standard ceramides were taken as their objective, aiming at elucidating the molecular structures within this complex lipid class. Few had mentioned the contaminants in cell extracts, because other lipid classes were not detected at the specific wavelength used for detecting only ceramide derivatives during UV or fluorescent derivatization analysis. The ELSD, a semi-universal detector, can detect all solutes that cannot evaporate. Thus, full separation of ceramides with other possible contaminants was required for qualification and quantitation. Mild alkaline hydrolysis treatment was performed to degrade most glycerides and phospholipids so that the lipid classes could be simplified. These two classes of lipids were hydrolyzed during the alkaline treatment process. The resulting products became water-soluble or they were formed as fatty acid methyl esters, which have little polarity, and they were readily separated from ceramides. Ceramides themselves were reported to be stable under the mild alkaline treatment (1 *M* methanolic KOH at 65 °C for at least 30 min [24]). Our previous experiment on the standard ceramides also confirmed its stability under such mild NaOH hydrolysis. But besides ceramides and sphingolipids, it was found that yeast sterol, namely ergosterol, was the most potential interference that may significantly disturb the assay of ceramides (Fig. 2A). Ergosterol is the predominant sterol in yeast, it could be stored in 2 to 3% cell dry weight, and most ergosterol is located in the plasma membrane. Despite its nonpolar moiety, a hydroxyl group on the rings can provide enough polarity similar to that of ceramides. It also has almost the same solubility as ceramides in most organic solvents, and is readily extracted out of the plasma membrane during the lipid extraction step. Besides, ergosterol is also resistant to NaOH hydrolysis, thus could not be eliminated before the HPLC injection. During the previous experiments on the silica column using a mobile phase of a classical isocratic ratio with chloroform–ethanol (90:10), we found that ergosterol could not be separated from ceramide 4 or ceramide 5, the ergosterol peaked at 5.90 min, while ceramide 4 and ceramide 5 at 6.5 min and 7.3 min, respectively. If the concentration of cell extracts increased, the peak of ergosterol would be so broad that it covered the ceramide peaks. However, when cell extract concentration was reduced to avoid broadening the ergosterol peak, ceramide contents were too low to be detected. Thus, incomplete separation of ceramides and ergosterol would make the quantitation impossible (Fig. 2B).

Other methods including the application of different mobile phases in HPLC on a silica column, small column pretreatment, and TLC (data not shown), failed to eliminate the interference of ergosterol in ceramide analysis. Thus, how to eliminate the ergosterol disturbance was critical for accurate ceramide analysis.

3.2. Separation of ceramides on a CN column

Ceramides and other contaminants were separated on a silica column under isocratic mode or gradient elution schemes. The isocratic mode based on the McNabb method [21] could not give satisfactory separation for ergosterol and ceramides (Fig. 2B). The gradient process was programmed based on Wells's experiment [11]. The program could separate ceramide 4, ceramide 5 and ergosterol well from each other (Fig. 2C). However, it was difficult to re-equilibrate the column again under the gradient elution program, resulting in poor reproducibility. In addition, long equilibration time and the high cost of the eluting solvent were also a problem for the assay based on the silica column. These troubles may be a result of the heterogeneous surface of silica columns. The state of column activation changed during the gradient elution and reactivation required a relatively long time to re-equilibrate for the next injection. Thus the assay method using a silica column was not convenient enough for the quantitation purpose. On the other hand, the silica column was a strong adsorbent, it could intensively retain polar lipids from cellular extracts. The silica column was purged thoroughly with ethanol every time after ~15 injections, yet the separation efficiency decreased after about 100 injections. It appeared that the silica column was vulnerable for the routine analysis of

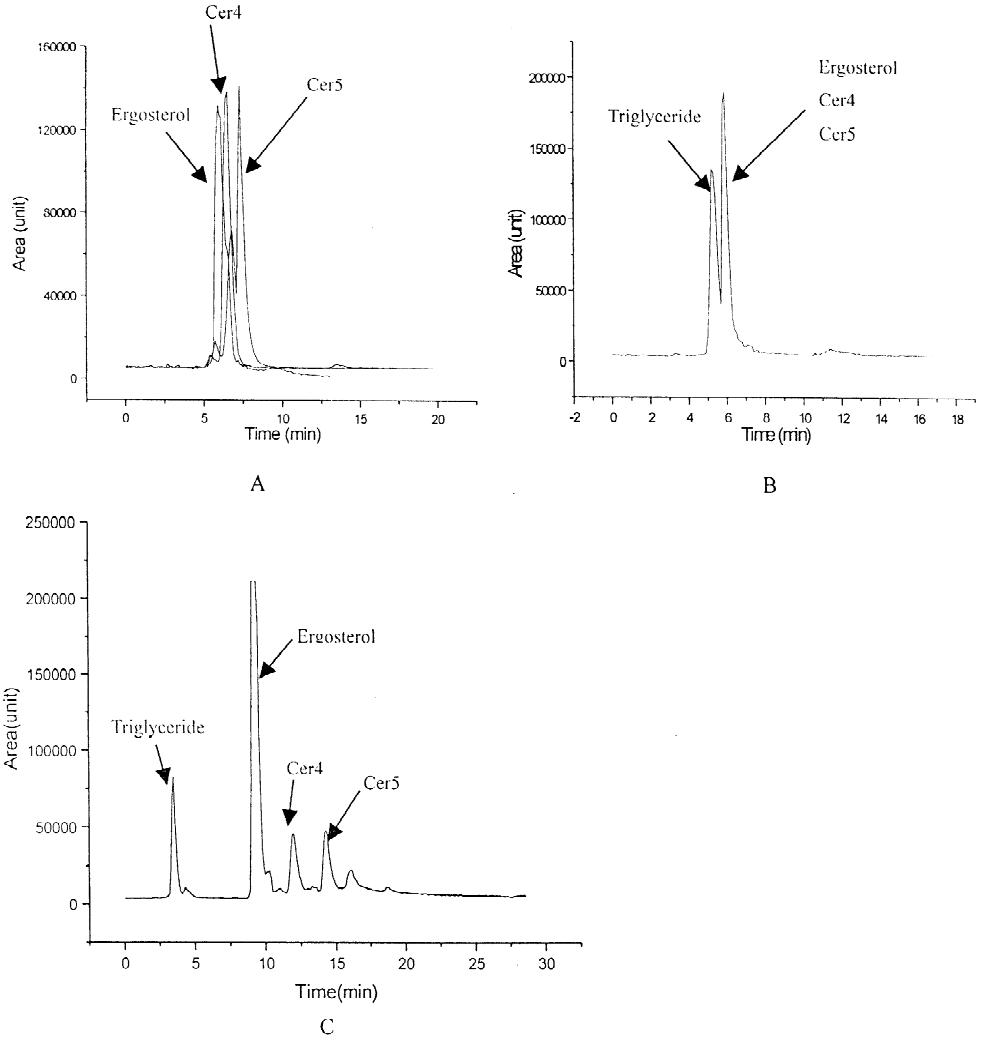


Fig. 2. Chromatogram of ceramide standard and lipid extracts of *Candida lipolytica* 33M on the silica column under isocratic or gradient elution program. (A) Isocratic elution (chloroform–ethanol (90:10), flow-rate 0.6 ml/min), multi-injections of standard ergosterol, ceramide 4 (Cer4), ceramide 5 (Cer5), showing the incapability of the silica column to separate these three substances. (B) Under the isocratic elution program, the cell lipid extracts of strain 33M on the silica column. There was no separation between interfering lipids and ceramide, thus making quantification and quantitation impossible. (C) Gradient elution, chloroform equilibrated for 1 min, followed by a 25-min linear gradient between chloroform and chloroform–ethanol (75:25). Then the column was re-equilibrated for another 10 min for the next injection (flow-rate 1.0 ml/min). Under such conditions, ceramide 4 (Cer4), ceramide 5 (Cer5), ergosterol, and triglycerides from cell lipid extracts were satisfactorily separated. The reproducibility was very poor. Column: Hypersil silica (46×250 mm, 5 μm). ELSD conditions: temperature, 40 °C; air pressure, 3.5 bar; gain, 8.

ceramides although it has been used for assaying other biological samples.

The CN column was a special bonded phase column based on silica. It worked under normal or reversed-phase, depending on what kind of solvent was applied. It was relatively easy to equilibrate or clean the polar contaminants retained in the column, since its surface adsorption ability was much weaker than that of the silica column. It also provided specific selection criteria for substances. The CN

column under the isocratic elution program was found to effectively separate ergosterol from ceramide standards (Fig. 3A).

Currently, many studies utilize ceramide standards from Sigma. However, these ceramides were obtained from decomposition of sphingomyelin of bovine brain, and the resulting ceramide molecules contain sphingosine instead of phytosphingosine. Thus, the validity of such ceramides as standards was questionable for the analysis of yeast-originated ceramides, because it had been widely acknowledged that phytosphingosine was the backbone of yeast ceramides [1], thus they have greater polarity over the bovine-originated molecules. The structures of ceramides provided by Cosmoferm were much more like those found in yeast (Fig. 1). Non-hydroxy FA ceramides (CerIII) coeluted with ceramide 4, both peaked at 14.1 min. Hydroxyl FA-containing ceramides (CerIV), peaking at 20.2 min on the HPLC–ELSD chromatogram, always eluted earlier than the second peak of ceramide 5, which peaked at 28.5 min (Fig. 3A). It should be noted that CerIII and CerIV were not single molecules, their FA sides contain carbon chains with different lengths, ranging from 16 to 24 [25], which explained the widening of the CerIV. This may also explain the broad peak width of the ceramide from yeast, which was reported to contain an FA carbon side chain ranging from 16 to 26 [1]. From these peak locations on the profile chromatogram, we could deduce that the use of mammalian original ceramides was not appropriate for the analysis of yeast ceramides; in our further experiments, only ceramides from Cosmoferm were utilized as standards. The first peak on the chromatogram of ceramide 5 was the isomer form of this molecule. We had utilized preparative TLC to separate the two corresponding bands, products purified from the two bands were subjected to mass spectroscopy (MS) study. They both gave m/z ratio at 600 under proper conditions. The lower

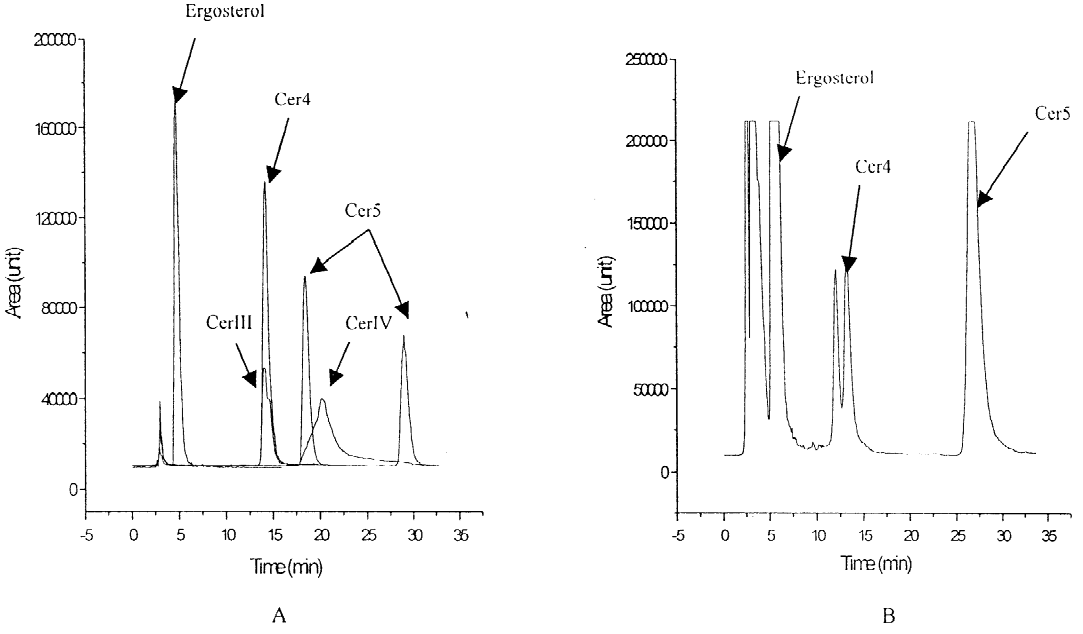


Fig. 3. Chromatogram of ceramide standard and lipid extracts of *Candida lipolytica* 33M on the CN column under isocratic elution program. (A) Multi-injections of standard ergosterol, ceramide 4 (Cer4), ceramide 5 (Cer5), ceramide III (CerIII), ceramide IV (CerIV) on the CN column, showing the capability of the column to separate those lipids. CerIV from Sigma was not suitable for identification of yeast-originated ceramides as CerIV eluted as a broad peak and faster than Cer5, which has a nearly identical structure to that of yeast ceramides. The two peaks of Cer5 were isomers. (B) Cell lipid extracts of strain 33M on the CN column. There was good separation between interfering lipids and ceramide, thus making quantification and quantitation possible. Column: Alltima CN (4.6×150 mm, 5 μm, Alltech). Mobile phase, hexane–ethanol (99:1); flow-rate, 1.0 ml/min. ELSD conditions: temperature, 40 °C; air pressure, 3.5 bar; gain, 8.

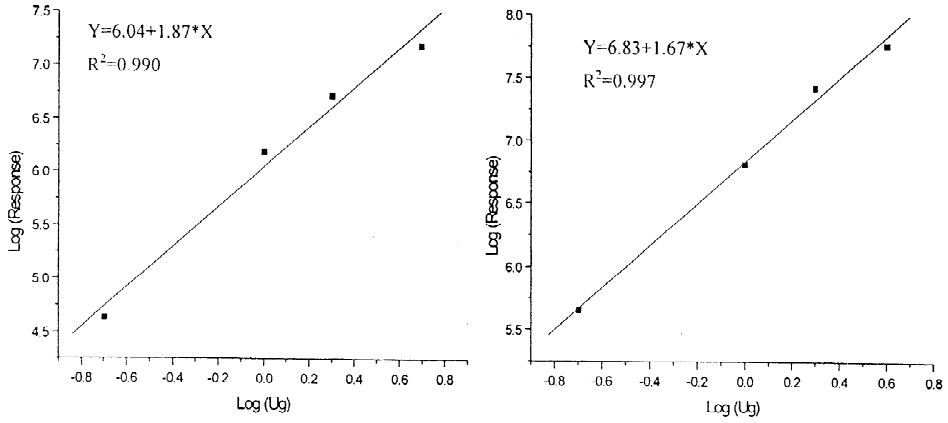
polarity of the first peak in ceramide 5 was supposed to be due to the fact that its hydroxyl group on the FA backbone was squeezed inside the molecules and could not contact with the surface of the CN or silica column, reducing its polarity to near that of ceramide 4. The area ratio of the first and second peak was always about 55:45, based on HPLC–ELSD at various concentrations. Since we only detected substances in yeast corresponding to the second peak in ceramide 5, so we excluded the first one in our further experiments. The standard curves were based on the second peak in ceramide 5, counting its amount as 45% of the total ceramides. Under isocratic elution on the CN column, the ergosterol, which peaked at 4.7 min, was readily resolved from ceramides, thus the large quantity of ergosterol would not interfere with ceramide analysis. Other possible contaminants, such as free fatty acids, minor amount of triglycerides and sterol esters all eluted between 3 and 5 min, close to ergosterol, far ahead of ceramides. In addition, these contaminants except for sterol esters could be destroyed during the NaOH hydrolysis pretreatment step.

Yeast strain *Candida lipolytica* 33M was reported to produce ceramides up to 0.5–1.0% of cell dry weight [22]; it could be used as a model for the establishment of the ceramide HPLC–ELSD analytical method. A typical chromatogram of the cell extracts of 33M grown under optimal conditions is shown in Fig. 3B. Ceramides 4 and 5 were clearly separated from other lipids and for qualification and quantitation.

3.3. Quantitation of ceramides in yeast cells on a CN column

The isocratic elution program provided much better reproducibility over the gradient program; it also required less re-equilibration time. Thus the plotting of a standard curve for ceramides was possible. According to the theory of ELSD, the response area of a solute was not linear with that of the sample mass. On the contrary, they were linear only when both were logarithmic. Fig. 4 illustrates the standard curves for ceramide 4 and 5 under double logarithmic mode. Only the response area and the amount of the second peak in ceramide 5 were calculated. Under the optimized HPLC–ELSD conditions, the detection limit for both ceramide 4 and ceramide 5 were 0.4 μg under the described ELSD conditions. Yet the optimal amount for quantitation should be above 0.8 μg.

To examine the efficiency and completeness of the total extraction and quantitation process, 1.2 l fermentation broth containing *C. lipolytica* 33M were divided into (ml) 2×100, 2×200, 2×300 broth. The



A B

Fig. 4. Standard curve of ceramide 5 (A) and ceramide 4 (B), respectively, under double logarithmic mode. Column: Alltima CN (4.6×150 mm, 5 μm, Alltech). Mobile phase, hexane–ethanol (99:1); flow-rate, 1.0 ml/min. ELSD conditions: temperature, 40 °C; air pressure, 3.5 bar; gain, 8. The amount of ceramide in ceramide 5 was counted as 45% of the total amount added because only one of the isomers was suitable for yeast ceramide 5 identification.

biomass of *C. lipolytica* was treated with the total extraction procedure described in Section 2. The amount of ceramide 4 and ceramide 5 increased with increasing volume of the fermentative broth in the ratio of 1.00:2.01:2.98, corresponding to 100, 200 and 300 ml of the broth. All parallel samples had a deviation smaller than 4.3%. Ceramide recovery during the extraction process exceeded 90%.

To ensure that polar lipids are removed out of the column, phosphatidylcholine (PC) was employed as an indicator since it was strongly retained on the silica column. Ethanol was applied to elute the polar lipid. When the flow-rate was maintained at 1.0 ml/min, the PC was eluted out of the CN column at about 5 min, indicating the easiness to clean the CN column when a series of samples (up to 35 samples or so) were analyzed using a normal mobile phase (hexane–ethanol, 99:1). Due to its short re-equilibration time, the column could be shifted back to the original mobile phase within 1 h for the next series of analyses.

Based on the separation and quantitation method established above, the contents of ceramides in several *Saccharomyces cerevisiae* strains were studied. It was found that ceramide contents in heat resistant strains 1912 was higher than that of normal strains 1408 and 4608, the ceramide 4 in all of these three *Saccharomyces cerevisiae* strains was too low to be detected, thus only ceramide 5 content was listed as 1.07‰, 0.73‰ and 0.15‰ of cell dry weight, respectively. The ceramide content in *Candida lipolytica* 33M was higher than the above three strains as ceramide 4 was more abundant; the contents of ceramides 4 and 5 were 0.96‰ and 1.39‰ of the cell dry weight. The concentration of ceramides in 33M was smaller than that reported in the literature [22] as the total ceramide content was reported as 4.76‰. This might be due to differences in the culture conditions (hexadecane was employed as carbon source compared with glucose in our study).

4. Conclusion

A simple and quantitative method based on HPLC–ELSD was developed for the routine analysis of ceramides in yeast cells. The CN column showed better separation ability and reproducibility over the generally adopted silica column. Under isocratic mode on the CN column, the contaminants would not interfere with the ceramide assay, and the column could be used for a series of injections followed by an easy cleaning step with ethanol to remove the polar lipids. The method can be applied in routine fermentative experiments to monitor the ceramide content in yeast.

Acknowledgements

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