methods

Microwave-mediated analysis for sugar, fatty acid, and sphingoid compositions of glycosphingolipids

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Abstract **For chemical characterization of glycosphingolipids, it is necessary to determine the chemical compositions of three constituents, i.e., sugars, fatty acids, and sphingoids. A new rapid analytical method is described using a one-pot reaction in a household microwave oven, producing sugars, fatty acids, and especially sphingoids free of by-products, from a single aliquot of a biological sample. Glycosphingolipids were hydrolyzed by microwave exposure with 0.1 M NaOH/CH_3OH for 2 min followed by 1 M HCl/CH_3OH for 45 s. The alkaline methanolysis step produced intermediate lysoglycosphingolipids virtually free of by-products such as the *O*-methyl ethers usually seen. The fatty acid methyl esters were extracted with n-hexane, and other reaction products were dried, taken up in aqueous alkaline methanol, and shaken with chloroform. Sphingoids partitioned into the organic phase under these conditions, whereas the sugar portion that partitioned into the aqueous phase was re-*N*-acetylated and remethanolyzed for 30 s by microwave exposure. Analysis of the profiles of glycosphingolipid constituents obtained using the microwave oven method showed that they were quantitatively and qualitatively comparable to those obtained by time-consuming conventional methods, which require reaction for several hours. Analysis of the three constituents, including analysis by gas chromatography, may be obtained within 1 day using the method described here.**—Itonori, S., M. Takahashi, T. Kitamura, K. Aoki, J. T. Dulaney, and M. Sugita. **Microwave-mediated analysis for sugar, fatty acid, and sphingoid compositions of glycosphingolipids. *J. Lipid Res.* 2004.** 45: **574–581.**

Supplementary key words acid hydrolysis • alkaline hydrolysis • lysoglycosphingolipid

Lipids are basic structural components of cell membranes in the animal kingdom. In Protostomia, the composition of these lipids is different from that in higher animals. In particular, membrane glycosphingolipids have different sugar species and aliphatic chain lengths, unsaturation positions and/or presence of hydroxyl group, as has been elucidated in our laboratory (1–12). To determine the glycosphingolipid structures, it is necessary to separate the three major components, i.e., sugars, fatty acids, and sphingoids, and to derivatize them before subjecting them to analysis by gas-liquid chromatography (GC) and combined gas-liquid chromatography-mass spectrometry (GC-MS). These chemical characterizations are usually achieved by separate procedures for sugars, fatty acids, and sphingoid moieties (13–15). It is important to develop a rapid analytical method that would consume smaller amounts of precious biological samples.

Methanolysis is a useful method of transesterification for obtaining methylglycosides and fatty acid methyl esters from glycosphingolipids (13, 14). Aqueous methanolysis is another useful method for obtaining sphingoid moieties from the same class of compounds (15). The conventional method requires reaction for several hours inside a tube in a boiling water bath or heating oven, which is a time-consuming step. Recently, reaction time for the esterification of fatty acids and the methanolysis of sugar components has been reduced using a microwave oven (10, 16–18). A high-yield preparation of lysoglycosphingolipids also has been developed with an alkaline hydrolysis by using a household microwave oven (19–21). Lysoglycosphingolipid lacking the fatty acyl group did not produce any *O*-methylsphingoids as artifacts, even by anhydrous metha-

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Abbreviations: CMS, ceramide monosaccharide; CTS, ceramide trisaccharide; ganglioside GM1, Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer; GC, gas-liquid chromatography; GC-MS, gas-liquid chromatography-mass spectrometry; Glc, glucose; GlcNAc, *N*-acetylglucosamine; HexNAc, *N*-acetylhexosamine; lyso-CMS, Galβ1-1sphingosine; lyso-CTS, GlcNAcβ1-3Manβ1-4Glcβ1-1sphingosine; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; Man, mannose; TMS, trimethylsilyl.

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nolysis (22). Taking these facts together, a new methodology of systematic structural analysis suggested itself and is presented in this paper (**Scheme 1**).

Although household microwave ovens are easily available, there are several key points to note in selecting an instrument and achieving the optimum conditions (10, 16–19). Here, we describe a method for applying a microwave-mediated reaction to glycosphingolipid structural analysis, including sphingoid moieties with an alkaline condition, by a one-pot reaction, and the confirmation of sugar and fatty acid components consequently obtained.

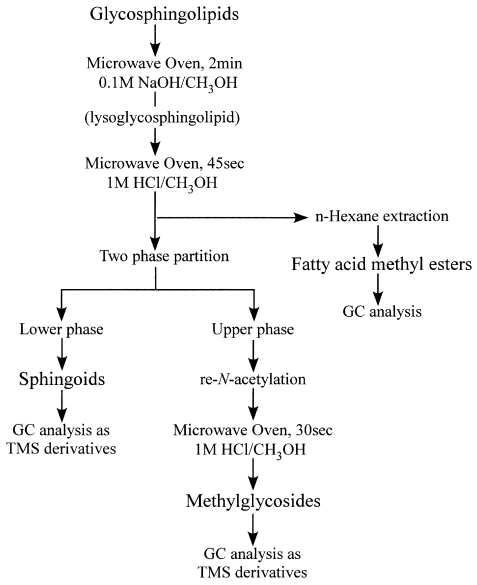
MATERIALS AND METHODS

Reagents

All solvents were purchased from Nacalai Tesque Co. (Kyoto, Japan) and were glass distilled before use. The solution of 0.1 M NaOH/CH_3OH was freshly prepared in our laboratory by dissolving a reagent grade NaOH (Nacalai Tesque Co., purity 96%) in distilled methanol, and 1 M HCl/CH_3OH was prepared by passing dry HCl gas through distilled methanol (23).

Glycosphingolipids

All glycosphingolipids isolated in our laboratory (1) were available for this experiment. These included ceramide monosaccharide (CMS, Galβ1-1Cer) and nonasaccharide [Fuc3Meα1-2Xyl3Meβ1-4(GalNAc3Meα1-3)Fucα1-4GlcNAcβ1-2Manα1-3(Xylβ1-2) Manβ1-4Glcβ1-1Cer] from the fresh-water bivalve *Hyriopsis schlegelii*; ceramide trisaccharide (CTS, GlcNAcβ1-3Manβ1-4Glcβ1-1Cer) and heptasaccharide (GlcNAcβ1-3Galβ1-3GalNAcα1-4GalNAcβ1-4GlcNAcβ 1-3Manβ1-4Glcβ1-1Cer) from the green bottle fly *Lucilia caesar*; phosphocholine-containing zwitterionic glycosphigolipid (cholinephosphoryl-6Galβ1-1Cer) from the earthworm *Pheretima hilgendorfi*; *N*-glycolyl-neuraminic acid-containing ganglioside (Araβ1-6Galβ1-4NeuGcα2-3Galβ1-4Glcβ1-1Cer) from the starfish *Asterina pectinifera*; and ganglioside GM1 [Galβ1-3GalNAcβ1-4(NeuAcα2-3)Galβ1-4Glcβ1-1Cer] from bovine brain.



Scheme 1. Systematic structural analysis of glycosphingolipids.

Microwave oven

Microwave ovens used in this experiment were ER-VS1 (Toshiba, Tokyo, Japan) and RE-Z3 (Sharp, Osaka, Japan). The technical specifications of both instruments were the same, as follows: power source 100 V, 60 Hz; power requirement 960 W; output power 500 W (High); frequency 2,450 MHz.

Preparation of sphingoids

Five to fifteen nanomoles of glycolipids (50 to 100 μg) were hydrolyzed in thick glass tubes (16 × 125 mm with Teflon-lined screw caps; Pyrex, Iwaki Glass Co., Tokyo, Japan) with 500 μl of freshly prepared 0.1 M NaOH/CH_3OH using a microwave oven (Toshiba, Model ER-VS1) attached to a GraLab Timer (Dimco-Gray Co., Dayton, OH). The samples were exposed to the maximum power (500 W) of the microwave oven for 1 to 3 min. For reasons of safety, the glass tubes were exposed one by one, each being enclosed in a large polypropylene plastic container. After hydrolysis, the samples were cooled to room temperature inside the microwave oven. Without evaporation or separation, the hydrolysates were methanolyzed by adding freshly prepared 1 M HCl/CH_3OH (300 μl) to the tubes and using the microwave oven at the maximum power for 45 s. For comparison, identical samples were methanolyzed with 200 μl of 1 M aqueous methanolic HCl in a conventional oven at 70°C for 18 h (14). The fatty acid methyl esters produced were extracted three times with 500 μl of n-hexane. The methanolic phase that remained was evaporated to dryness for deacidification under N_2 gas. The residue containing sphingoids and methylglycosides was dissolved in 600 μl of methanol-0.1 M NaOH (4:3, v/v), and 720 μl of chloroform was then added. After vigorous agitation, the two phases were separated by centrifugation (3,000 rpm, 5 min) and the upper phase containing methylglycosides was removed. The lower phase containing sphingoids was washed twice with 400 μl of methanol-water (1:1, v/v) and evaporated to dryness under N_2 gas (24).

Preparation of lysoglycosphingolipid

After alkaline hydrolysis (microwave oven, 2 min; 0.1 M NaOH/CH_3OH), the samples were acidified by the addition of two drops of 6 M HCl. One milliliter of methanol was added, and excess salts were separated by centrifugation (3,000 rpm, 5 min). The methanol phase was transferred to a fresh tube, evaporated

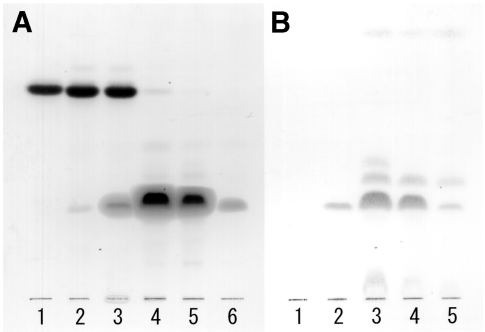


Fig. 1. Microwave exposure time course of reaction for the preparation of lysoglycosphingolipid from ceramide monosaccharide (CMS) of the fresh-water bivalve *H. schlegelii* as determined by thin-layer chromatography. A: Lane 1, intact CMS; lane 2, 1 min; lane 3, 1 min and 30 s; lane 4, 2 min; lane 5, 2 min and 30 s; lane 6, 3 min. B: Lane 1, 1 min; lane 2, 1 min and 30 s; lane 3, 2 min; lane 4, 2 min and 30 s; lane 5, 3 min. The plates were developed in chloroform-methanol-water (60:40:10, v/v/v). The spots were visualized with orcinol-H_2SO_4 reagent (A) and ninhydrin reagent (B).

TABLE 1. Yields (%)[a] of sphingoid and fatty acid from ceramide monosaccharide by anhydrous methanolysis, aqueous methanolysis, and the new method

	Anhydrous[b]	Aqueous[c]	Alkaline and Scid[d]
Sphingoid	nd[e]	100 ± 19	104 ± 6
Fatty acid	100 ± 4	95 ± 3	100 ± 6

[a] Yields were calculated as 100% using the d18:1 sphingoid gas-liquid chromatography peak area obtained after aqueous methanolysis for sphingoid, and total peak areas of fatty acids after anhydrous methanolysis using acid propyl ester as internal standard.

[b] 1 M HCl/CH_3OH for 45 s using a Toshiba ER-VS1 microwave oven.

[c] 1 M HCl in water/CH_3OH for 18 h at 70°C in a conventional oven.

[d] 0.1 M NaOH/CH_3OH for 2 min and 1 M HCl/CH_3OH for 45 s using a Toshiba ER-VS1 microwave oven.

[e] Not determined primarily because by-product was observed.

to dryness under N_2 gas, and analyzed by thin-layer chromatography (TLC).

Preparation of fatty acid methyl esters

The fatty acid methyl esters were extracted with n-hexane as described above after methanolysis (microwave oven, 45 s; 1 M HCl/CH_3OH) and evaporated to dryness under N_2 gas.

Preparation of methylglycosides

Two drops of 6 M HCl were added to the upper phase containing methylglycosides partitioned as described above, and the solution was evaporated to dryness under N_2 gas. One milliliter of methanol was added to the residue, excess salts were removed by centrifugation (3,000 rpm, 5 min), and the methanolic solution was transferred to a fresh tube. Re-*N*-acetylation for hexosamines was performed by the addition of 10 μl of pyridine and 50 μl of acetic anhydride at room temperature for 30 min, followed by evaporation to dryness under N_2 gas (25). Remethanolysis was performed by the addition of 200 μl of 1 M HCl/CH_3OH, exposure to the microwave oven for 30 s, and evaporation.

GC and GC-MS

Sphingoids and methylglycosides were trimethylsilylated with pyridine-hexamethyldisilazane-trimethylchlorosilane (9:3:1, v/v/v) at 60°C for 30 min (26). Their trimethylsilyl (TMS) derivatives were partitioned by 1 ml of chloroform and 4 ml of water. The water phase was replaced three times, and the chloroform phase was evaporated under N_2 gas. An aliquot of the residues was injected and analyzed by a gas chromatograph. Stearic acid propyl ester was used as internal standard for sphingoid TMS derivatives and fatty acid methyl esters. All of these derivatives were analyzed by using a Shimadzu GC-18A gas chromatograph and a capillary column of Shimadzu HiCap-CBP 5 (0.22 mm × 25 m, Shimadzu Co., Kyoto, Japan). Electron impact mass spectra were taken using a Shimadzu GCMS-QP 5050 gas chromatograph-mass spectrometer with the same capillary column under the following conditions: ionizing voltage, 70 eV; ionizing current, 60 μA; interface temperature, 250°C; injection port temperature, 240°C; helium gas pressure, 100 kPa. Oven temperatures were programmed as follows: sphingoid TMS derivatives, 210°C to 230°C (2°C/min) for GC, 80°C (2 min) to 180°C (20°C/min) to 240°C (4°C/min) for GC-MS; fatty acid methyl esters, 170°C to 230°C (4°C/min) for GC, 80°C (2 min) to 180°C (20°C/min) to 240°C (4°C/min) for GC-MS; methylglycoside TMS derivatives, 150°C to 230°C (2°C/min) for GC, 80°C (2 min) to 180°C (20°C/min) to 240°C (4°C/min) for GC-MS.

TLC

Silica gel 60 precoated plates (Merck KgaA, Darmstadt, Germany) were developed with chloroform-methanol-water (60:40:10, v/v/v). Glycolipids were visualized by spraying with orcinol-H_2SO_4 reagent (27), and the free amino group was visualized by spraying with ninhydrin reagent (28) followed by heating at 110°C.

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry

Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) analyses were performed using a Shimadzu/KRATOS KOMPACT MALDI I mass spectrometer (Shimadzu Co.) equipped with a Workstation SPARC station (Sun Microsystems, Inc., Newark, CA) operating in positive-ion linear mode. Ions were formed by a pulsed UV laser beam (N_2 laser, 337 nm; 3 ns-wide pulses/s). The matrix used was 7-amino-4-methyl-coumarin (coumarin 120; Sigma, St. Louis, MO). External mass calibration was provided by the $[M + H]^+$ ions of ceramide mono- to nonasaccharides (699 to 2,201 molecular weight) prepared from the green bottle fly *L. caesar* (7, 10).

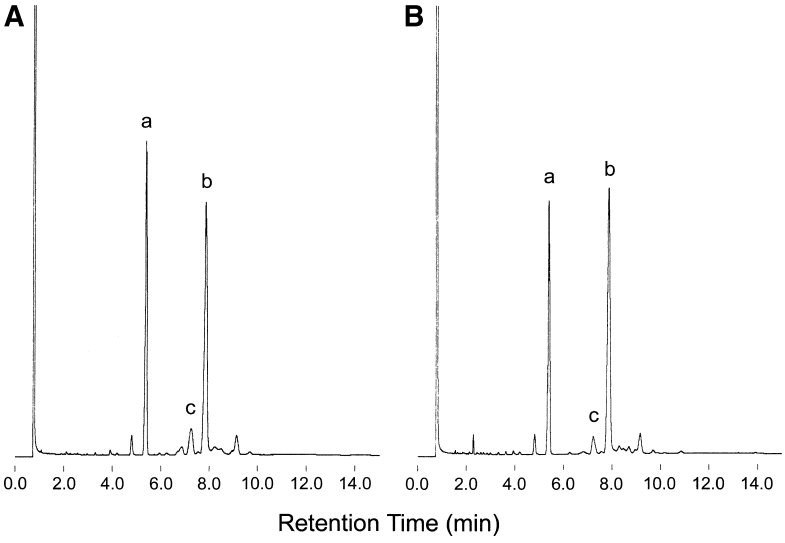


Fig. 2. Gas chromatogram profiles of sphingoid from CMS of the fresh-water bivalve *H. schlegelii*. A: Chromatogram of the sphingoid obtained by aqueous methanolysis, hydrolyzed with 1 M HCl in water/CH_3OH for 18 h at 70°C in a conventional oven (15). B: Chromatogram of the sphingoid obtained by the method presented in this article. Peak a, stearic acid propyl ester as internal standard; peak b, octadeca-4-sphingenine; peak c, by-products from sphingoids. Sphingoids were analyzed as trimethylsilyl (TMS) derivatives.

RESULTS

Microwave exposure time

To determine an efficient microwave exposure time, the simplest glycosphingolipid, CMS from the fresh-water bivalve, was hydrolyzed with 0.1 M NaOH/CH_3OH using a microwave oven. **Fig. 1** shows the time course of the amide bond hydrolysis analyzed by TLC. Little Galβ1-1sphingosine (lyso-CMS) was observed within 1 min 30 s reaction time (Fig. 1A, lanes 2 and 3 and 1B, lanes 1 and 2). At 2 to 3 min reaction times, however, substantial conversion was detected by both orcinol-H_2SO_4 reagent and ninhydrin reagent (Fig. 1A, lanes 4–6 and 1B, lanes 3–5). The ninhydrin-positive spot migrating ahead of lyso-CMS (barely visualized by orcinol-H_2SO_4 reagent) seems to be the sphingoid directly formed under this condition (Fig. 1B, lanes 3–5). Prolonging the reaction time for 2 more min led to a lower yield of lyso-CMS due to degradation of products. Using 0.05 M NaOH/CH_3OH, mostly intact CMS was observed after an exposure time of 2 min (data not shown). Therefore, the optimal reaction time for the alkaline hydrolysis was found to be 2 min with 0.1 M NaOH/CH_3OH. It should be emphasized that this hydrolysis was observed using a Toshiba ER-VS1 oven. Using a Sharp RE-Z3 oven, no lyso-CMS was produced using an even longer reaction time (data not shown). Although no performance difference can be inferred from the technical specification sheets provided with the Toshiba and Sharp instruments, the Toshiba microwave oven is suitable for this reaction. This is a key point for selection of an instrument.

Moisture contamination in the hydrolysis reagent

The alkaline hydrolysis solution used in this experiment contains hygroscopic reagents. We examined the effect of moisture contamination by the addition of water to the solution. Complete hydrolysis occurred with 0.25% water in methanol, the same as observed with distilled methanol alone as a solvent (data not shown). With 0.5% water in methanol, unhydrolyzed CMS was observed, and using 1.5% water in methanol, no hydrolyzed product could be observed (data not shown). We have experienced complete hydrolysis using the freshly prepared alkaline solution in distilled methanol.

Yield of sphingoids

Lyso-CMS was produced from CMS with freshly prepared 0.1 M NaOH/CH_3OH using a microwave oven for 2 min at maximum power. Afterwards, sphingoids were prepared by hydrolysis with freshly prepared 1 M HCl/CH_3OH in the same tube using the microwave oven at the maximum power for 45 s, and were partitioned under alkaline conditions. Sphingoid TMS derivatives produced by this method were compared with those produced by the conventional method (1 M aqueous methanolic HCl in a conventional oven at 70°C for 18 h) by calculating areas on their respective GC chromatograms. The internal standard stearic acid propyl ester was used. The yield of

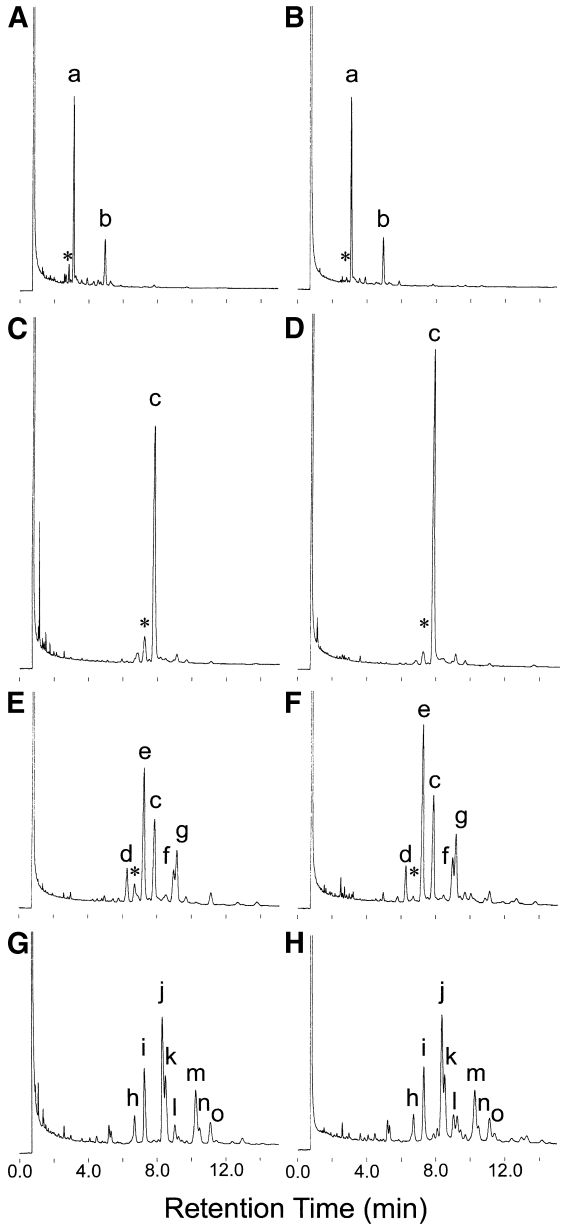


Fig. 3. Gas chromatogram profiles of various sphingoids from glycosphingolipids. A, C, E, G: Chromatograms of the sphingoid obtained by aqueous methanolysis, hydrolyzed with 1 M HCl in water/CH_3OH for 18 h at 70°C in a conventional oven (15). B, D, F, H: Chromatograms of the sphingoid obtained by the method presented in this article. A, B: Ceramide heptasaccharide isolated from the green bottle fly *L. caesar*. C, D: Ceramide nonasaccharide isolated from the fresh-water bivalve *H. schlegelii*. E, F: Phosphocholine-containing, zwitterionic glycosphingolipid isolated from the earthworm *P. hilgendorfi*. G, H: Ganglioside isolated from the starfish *A. pectinifera*. Peak a, C_{14}-sphingosine; peak b, C_{16}-sphingosine; peak c, C_{18}-sphingosine; peak d, C_{17}-sphingosine; peak e, branched C_{18}-sphingosine; peak f, branched C_{19}-sphingosine; peak g, C_{19}-sphingosine; peak h, iso-C_{16}-phytosphingosine; peak i, C_{16}-phytosphingosine; peak j, iso-C_{17}-phytosphingosine; peak k, anteiso-C_{17}-phytosphingosine; peak l, C_{17}-phytosphingosine; peak m, iso-C_{18}-phytosphingosine; peak n, anteiso-C_{18}-phytosphingosine; peak o, C_{18}-phytosphingosine; peaks *, by-products from sphingoids. Sphingoids were analyzed as TMS derivatives.

sphingoids was quantitatively slightly higher (104%) and the error was less (n = 3) than in the conventional method (**Table 1**). This shows that the one-pot reaction system is fully reproducible. Cleavage of glycosphingolipids with anhydrous methanolic HCl results in the production of considerable quantities of secondary products from sphingoids, such as *O*-methyl ethers (17, 22). Although a modified reagent for methanolysis (conventional method), containing methanol, water, and HCl, reduces the yield of these by-products to low levels, by-products can nevertheless be detected (15). Theoretically, no by-product sphingoids are produced from lysoglycosphingolipids with anhydrous methanolysis (22). Our results show much fewer by-products as artifacts, compared with the conventional method (**Fig. 2A**, B, peak c). This one-pot reaction system not only reduces the reaction time to 2 min and 45 s from 18 h, but also produces better yields and fewer by-products. That is, from as little as 20 μg and up to 500 μg of CMS (~3 to ~70 nmol), largely the same gas chromatogram patterns of the sphingoid, as well as a similar by-product/product ratio, were obtained from the same hydrolysis condition.

Application to various types of sphingoids and glycosphingolipids

Shorter chain length sphingoids were derived from ceramide heptasaccharide isolated from the green bottle fly *L. caesar*, which contains a moderate-length sugar chain. As shown in **Fig. 3**, d14:1 and d16:1 sphingoids comparable to those found using the conventional method were detected (Fig. 3A, B). Using ceramide nonasaccharide isolated from the fresh-water bivalve *H. schlegelii*, which contains a longer sugar chain, d18:1 sphingoid was detected (Fig. 3D). Neither sphingoid chain length nor sugar chain length in glycosphingolipids appears to affect the yield of sphingoid or the reactions of alkaline hydrolysis and methanolysis. In the phosphocholine-containing zwitterionic glycosphingolipid isolated from the earthworm *P. hilgendorfi*, the sphingoid species contains branched structures. Accordingly, all sphingoid species in this lipid were detected by the one-pot reaction system, independent of the attached group or substituent on the sugar molecule (Fig. 3F). Ganglioside isolated from the starfish *A. pectinifera* contains *N*-glycolylneuraminic acid as its sugar component and phytosphingoids as sphingoid species. Every sphingoid species in this ganglioside was detected, and no degradation of phytosphingoid was found using this system (Fig. 3H). Similarly, in the ubiquitous ganglioside GM1, isolated from bovine brain, typical d18:1 and d20:1 sphingoids were detected (data not shown). In our experience, every type of sphingoid species in glycosphingolipids could be determined by this one-pot reaction system, independent of the differences of sugar structures and the types of sphingoid species. Also, it should be noted that the yield of by-products is trivial in most cases.

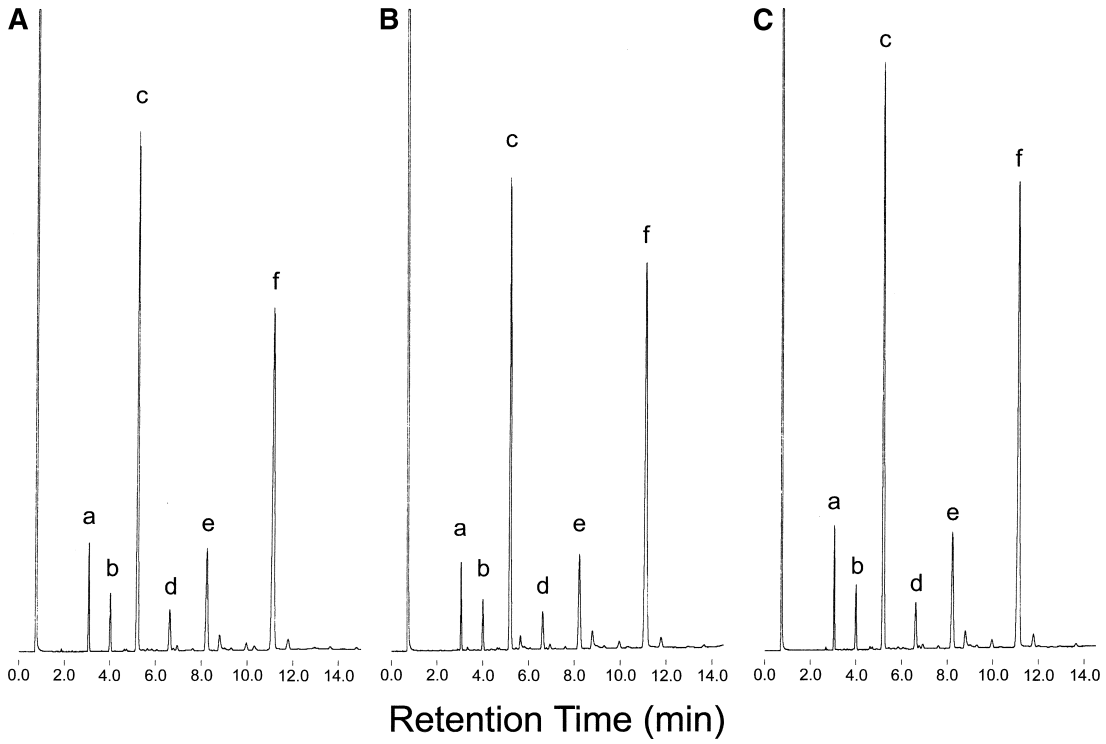


Fig. 4. Gas chromatogram profiles of fatty acid methyl esters from CMS of the fresh-water bivalve, *H. schlegelii*. A: Chromatogram of the sphingoid obtained by anhydrous methanolysis, hydrolyzed with 1 M HCl/CH_3OH for 45 s by a Toshiba ER-VS1 microwave oven. B: Chromatogram of the sphingoid obtained by aqueous methanolysis, hydrolyzed with 1 M HCl in water/CH_3OH for 18 h at 70°C in a conventional oven (15). C: Chromatogram of the sphingoid obtained by the method presented in this article. Peak a, C14:0; peak b, C15:0; peak c, C16:0; peak d, C17:0; peak e, C18:0; peak f, stearic acid propyl ester as internal standard.

Yield of fatty acid methyl ester

Methanolysis, which hydrolyzes the amide bond and esterifies the resulting free fatty acid, produces fatty acid methyl esters from anhydrous methanolic HCl by boiling in water for 3 h, or by microwave exposure for 1 min (13, 14, 18). In the first step of this one-pot reaction system, alkaline hydrolysis of glycosphingolipids cleaves the amide bond, resulting primarily in free fatty acids; no fatty acid methyl esters are detected by GC at this step (data not shown). Fatty acid methyl esters are produced during the subsequent acid methanolysis required for cleavage of glycosyl bonds attached to sphingoids. The resulting fatty acid methyl esters are easily extracted into n-hexane. Their yields by the new method are compared with results obtained using traditional methods (**Fig. 4** and Table 1), and are quantitatively and qualitatively comparable to results by other methods.

Carbohydrate constituent analysis

Under the optimal reaction condition for sphingoid analysis, cleavage of an acetyl group from a lyso-CMS [*N*-acetylhexosamine (HexNAc)] residue could occur (20). An *N*-acetylglucosamine (GlcNAc)-containing glycosphingolipid, CTS (GlcNAcβ1-3Manβ1-4Glcβ1-1Cer) isolated from the green bottle fly *L. caesar*, was hydrolyzed with 0.1 M NaOH/CH_3OH using a microwave oven and analyzed by MALDI-TOF MS (data not shown). The product was represented mainly by two $[M + H]^+$ ions differing by 28 *m/z* units and corresponding to the molecular species containing d14:1 and d16:1 sphingoids. The $[M + H]^+$ ions at *m/z* 729 and 757 differ by 42 *m/z* units [− (CH_3CO) + H] from the calculated $[M + H]^+$ ions of lyso-CTS at *m/z* 771 and 799, respectively. These $[M + H]^+$ ions at *m/z* 729 and 757 correspond to deacetyl-lyso-CTS and show the lack of both acetyl and fatty acyl groups from native CTS.

In the one-pot reaction system, without re-*N*-acetylation of deacetyl-lyso-CTS, only glucose (Glc) could be detected still attached to its sphingoid (**Fig. 5A**). After 30 min re-*N*-acetylation (25), the sugar portion was cleaved using microwave exposure for 30 s and analyzed by GC as the TMS derivatives. As shown in Fig. 5B, the sugar constituents of CTS, namely mannose (Man), Glc, and GlcNAc, were detected in molar ratios identical to those found in traditional methanolysis as 1:1:1 (**Table 2**). In this traditional methanolysis method, complete cleavage of glycosyl bonds was achieved, whereas the amide bond on HexNAc was stable and therefore required no re-*N*-acetylation after methanolysis (S. Itonori et al., unpublished observations).

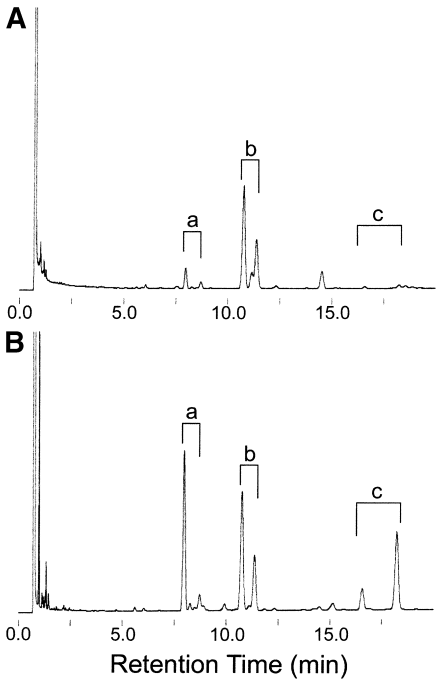


Fig. 5. Gas chromatogram profiles of sugar moieties from ceramide trisaccharide isolated from the green bottle fly *L. caesar*. A: Chromatogram of the methylglycoside TMS derivatives obtained by following the sequential reactions: alkaline hydrolysis, anhydrous methanolysis, and TMS derivatization. B: Chromatogram of the methylglycoside TMS derivatives obtained by the method presented in this article. Peaks a, mannose; peaks b, glucose; peaks c, *N*-acetylglucosamine.

DISCUSSION

The microwave oven has been used to speed up synthetic reactions in the preparation of various organic compounds (29–31). In the laboratory, the microwave generator used early on was a laboratory-grade instrument; for example, in 1993, Lagana et al. (32) described the analysis of sialic acids using the Microwave Digestion System (CEM Co.). More recently, household microwave ovens have been used for chemical analysis of protein, lipid, and sugar structures in the laboratory (33–38). In our laboratory, a household microwave oven has been used for fatty acid compositional analysis of phospholipids and glycosphingolipids, as well as for carbohydrate structural analysis of glycosphingolipids (10, 18).

In determining the best hydrolysis conditions, it is critical to establish the optimal reaction time. Although the typical household microwave oven has a power control, this switch permits only on/off control. Therefore, it is necessary to find the optimum reaction time point at fixed maximum power. We describe here glycosphingolipids that were hydrolyzed by microwave exposure with 0.1 M NaOH/CH_3OH for 2 min followed by 1 M HCl/CH_3OH for 45 s. Taketomi and colleagues (19–21) have described the preparation of lysoglycosphingolipid after exposure for 2 min (likewise found by his group to be op-

TABLE 2. Sugar compositions of ceramide trisaccharide by anhydrous methanolysis and the new method

	Mannose	Glucose	*N*-acetylglucosamine
Anhydrous	0.89	1.00	0.62
Alkaline and acid	0.84	1.00	0.71

Methanolysis conditions are the same as for Table 1. Glucose is expressed as 1.00.

timal) using a Toshiba ER-V microwave oven. On the other hand, it should be noted that there is a difference in microwave generating capacity that cannot be estimated from data sheets of the appliances. For example, the Sharp RE-Z3 oven could not produce lysoglycosphingolipid from glycosphingolipid under alkaline hydrolysis conditions even after prolonging the exposure time to 3 min. From another point of view, at the 1 min time point using the Sharp RE-Z3 oven, the recovery of fatty acid methyl ester was less than that found when the Toshiba was used for 30 s, whereas some sugar components could be detected only by the latter microwave-mediated method (10, 18). This was especially true of xylose and its methyl derivatives, which occur in Protostomia glycosphingolipids, due to the fact that the reaction is milder using the microwave method than that seen using the conventional method, and sugar structures are better retained. This might be considered a drawback to using household microwave ovens, but it is nevertheless possible to locate a cheap and convenient oven that is acceptable for alkaline hydrolysis.

We chose the concentration of NaOH in methanol to be 0.1 M. At a lower concentration, i.e., 0.05 M NaOH in distilled methanol, mostly intact CMS was observed after 2 min microwave exposure. A concentration of 0.1 M NaOH in distilled methanol is approximately saturated, because particles of NaOH could be seen in the solution. In other words, when a clear solution of 0.1 M NaOH in distilled methanol is seen, moisture contamination of the reagent should be suspected. This one-pot reaction system requires alkaline hydrolysis followed by acid methanolysis. Although the latter is not affected by salt or water (15, 37, 39), the presence of these in the solution should be kept to a minimum for subsequent isolation of sphingoids and carbohydrates by partioning under alkaline conditions. All of these considerations dictated our choice of 0.1 M NaOH as the optimum concentration in this system.

Various methods involving alkaline conditions have been reported for the preparation of lysoglycosphingolipids, mostly for the preparation (from gangliosides) of deacetyl lysogangliosides lacking both fatty acyl groups and acetyl groups on neuraminic acids and hexosamines (22, 40–42). A later, improved method described the preparation of lysogangliosides by use of a one-pot reaction (43). Recently, methods have been reported for preparing various kinds of lysoglycosphingolipids by microwave exposure (19–21). Using this technique, alkaline hydrolysis of amide bonds can be controlled by careful timing, resulting in lysoglycosphingolipids and deacetyl lysoglycosphingolipids. In our experiments, primarily deacetyl lysoglycosphingolipids were observed; but from an analytical point of view, deacylation, including deacetylation of carbohydrate moieties, does not affect the analysis of the sphingoid moiety. For carbohydrate analysis, however, re-*N*-acetylation is required for the cleavage of hexosamine-containing glycosyl bonds. Once the acetyl group has been cleaved from HexNAc, the residual hexosamine glycosyl bonds are extremely resistant to acid hydrolysis, because of protonation located close to the glycosyl bond (22). Man and glucosamine were practically undetectable at their corresponding retention times, because at this point, they exist primarily as the GlcN-Man disaccharide (Fig. 5A). However, for release of sphingoid, acid methanolysis is required after alkaline hydrolysis. It is therefore necessary that the sugar-containing residual moiety should be re-*N*-acetylated and remethanolyzed. The resistance of hexosaminyl glycosyl bonds to alkaline hydrolysis is useful for carbohydrate sequential analysis. In fact, the relative amounts of GlcNAc and Man observed in GC analysis in the absence versus the presence of re-*N*-acetylation implies the GlcNAc-to-Man link (Fig. 5A), and the method described here might thus help provide information regarding carbohydrate sequences in glycosphingolipids.

Without a separation of sugars and sphingoids, one-step analysis by GC of our reaction mixture could determine the products as *N*-acetyl sphingoid and methylglycoside TMS derivatives (44). However, the relative intensities of methylglycoside TMS derivatives compared with *N*-acetyl sphingoid TMS derivatives (2- to 4-fold higher, data not shown) complicate the assignment of peaks to various components. Also, fewer data are available for mass spectra of of *N*-acetyl sphingoids and their GC particulars. Therefore, we chose to separate sugar and sphingoid moieties and analyze them separately.

The overall conclusion drawn from this study of microwave-mediated reaction in glycosphingolipid analysis is that the method can be usefully applied for rapid qualitative analysis. Application of this method to analysis of phosphosphingolipids and inositol phosphate containing glycosphingolipids is in progress and will be published elsewhere.

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