methods

Confirmation of minor components of less polar neutral and acidic glycolipids in monkey brain tissue

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Abstract **Crude glycolipids, prepared without alkali treatment in advance, were separated into neutral and acidic glycolipids by DEAE-Sephadex A-25 (acetate form) column chromatography. Each glycolipid was further fractionated by a Silica gel 60-column chromatography. By matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with delayed ion extraction (DE MALDI-TOF MS) of the intact glycolipid fractions, the less polar neutral glycolipids were found to contain alkali-labile ester cerebrosides and Galβ-1-Diradylglycerols, whereas the less polar acidic glycolipids were found to contain alkali-labile ester sulfatide, HSO_3-3Gal-1-Diradylglycerols, and novel alkali-stable plasmalo-sulfatides and ester or plasmalo HSO_3-3Galβ-1-Diradylglycerols as minor components of glycolipids in monkey brain tissue. In conclusion, minor components of less polar neutral and acidic glycolipids in monkey brain tissue were confirmed as ester cerebrosides, Galβ-1-Diradylglycerols, ester sulfatides, HSO_3-3Galβ-1-Diradylglycerols, and novel plasmalo-sulfatides and ester or plasmalo HSO_3-3Galβ-1-Diradylglycerols by DE MALDI-TOF MS.—**Taketomi, T., E. Sugiyama, K-i. Uemura, A. Hara, H. Hidaka, M. Tozuka, T. Nakabayashi, and T. Katsuyama. **Confirmation of minor components of less polar neutral and acidic glycolipids in monkey brain tissue. *J. Lipid Res.* 2001.** 42: **873–885.**

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Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with delayed ion extraction (DE MALDI-TOF MS), improved with the new delayed-ion extraction technique, is useful for the confirmation of many glycolipids, of which the chemical structures are already known, because it offers the advantages of broad mass range, high sensitivity, high accuracy, and high resolution. We have confirmed several sphingoglycolipids, including gangliosides and lysosphingoglycolipids (1–7). In this study, targeting minor components of less polar neutral and acidic glycolipids, such as ester cerebrosides, plasmalo-cerebrosides, and ester sulfatides (8–15), of which the function and distribution in the nerve tissues still remain to be known, crude glycolipids prepared without alkali treatment in advance were separated into the neutral and acidic glycolipids by DEAE-Sephadex A-25 (acetate form) column chromatography. Each glycolipid was fractionated with Silica gel 60-column chromatography. The different fractions were directly and accurately analyzed by DE MALDI-TOF MS to identify certain kinds of glycolipids, depending on the mass to charge ratios (m/z) of their molecular ion signals, although they had in advance been checked by thin-layer chromatography (TLC). Also, after the mild alkali treatment of the different fractions to distinguish between the alkali-stable and labile glycolipids, the products derived from the neutral and acidic fractions were applied to DE MALDI-TOF MS. Minor components of alkali-labile ester cerebroside and Galβ-1-Diradylglycerols in the neutral glycolipids, as well as alkali-labile ester sulfatide, HSO_3-3Galβ-1-Diradylglycerols, ester or plasmalo HSO_3-3Galβ-1-Diradylglycerols, and alkali-stable plasmalo-sulfatide in the acidic glycolipids, were confirmed in monkey brain tissue. At the same time, their individual molecular species were clarified by DE MALDI-TOF MS.

MATERIALS AND METHODS

Materials

DEAE-Sephadex A-25 was purchased from Pharmacia-LKB (Sweden). Silica gel 60 and precoated TLC plates were obtained from Merck (Germany). 2,5-Dihydroxybenzoic acid as the matrix for MALDI-TOF MS was obtained from Sigma Chemical (USA). Other reagents were of analytical grade.

Abbreviations: DE MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with delayed ion extraction; 2,5-DHB, 2,5-Dihydroxy benzoic acid; LCB, long-chain base; m/z, mass to charge ratio; TLC, thin-layer chromatography; ester cerebroside, cerebroside fatty acid esters; ester sulfatide, sulfatide fatty acid esters; plasmalo-cerebroside, fatty aldehyde conjugate of cerebroside; plasmalo-sulfatide, fatty aldehyde conjugate of sulfatide.

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Fractionation of minor components from crude glycolipids prepared without alkali treatment in advance from total lipids

A monkey (*Macaca fuscata*) was housed and treated in accordance with the institutional guideline. A part of the brain tissue was available for the study. The cerebral white matter was homogenized with chloroform-methanol (C/M, 2:1 v/v) as 10 ml/g of the wet tissue and filtered through a sintered glass. After the residue was rehomogenized in the same volume of the same solvent, the extracts were combined and treated by Folch partition using one-fifth volume of water containing 0.88% KCl. The lower phase was evaporated to dryness under vacuum, and the residue was treated with acetone twice to remove cholesterol and simple lipids. The acetone-insoluble residue (about 500 mg) dissolved in chloroform-methanol-water (C/M/W, 30:60:8 v/v/v) was applied to a DEAE-Sephadex A-25 (acetate form) column (1 cm in diameter, 25 cm high) for the separation of neutral and acidic lipids. After the neutral lipids were thoroughly eluted with the same solvent, the acidic lipids were then eluted with C/M/0.8 M sodium acetate (30:60:8, v/v/v).

The acidic fraction was dialyzed to remove salt. After the neutral lipid fraction was evaporated to dryness, the residue (~400 mg) was dissolved in C/M (98:2 v/v) and rechromatographed on a Silica gel 60-column stepwise with C/M (98:2 v/v), (95:5 v/v), (90:10 v/v), and (2:1 v/v). The fractions collected into tubes were checked by TLC and directly applied to DE MALDI-TOF MS. The acidic lipid fraction (~86 mg) was dissolved in C/M (95:5 v/v), loaded on a Silica gel 60 (about 9 gm)-column (1 cm in diameter), and eluted first with about 100 ml of the same solvent and then with linear gradient (0.2%/min) in C/M/W (80:29:0.5 v/v/v) to (10:90:5 v/v/v) at 0.3 ml/min. The fractions collected into tubes were checked by TLC and were directly applied to DE MALDI-TOF MS.

Mild alkaline hydrolysis

For the determination of mild alkali-labile minor components of neutral and acidic glycolipids, ~1 mg of the sample was dissolved in 1 ml of 0.5 N NaOH in methanol and kept at 56°C for 60 min in a water bath. After the reaction mixture was cooled and acidified with 3 N HCl, it was mixed vigorously with hexane. After removing the upper phase containing fatty acids, the lower phase was evaporated to dryness. The residue was then partitioned with the theoretical upper phase (C/M/W, 3:48:47 v/v/v) and lower phase (C/M/W, 86:14:1 v/v/v) solution to remove salt. After centrifuging the mixed solution at 3,000 rpm for 10 min, the upper phase was discarded. The lower phase was evaporated to dryness, and if the residue still contained salt, the same partition was repeated. Finally, the residue was dissolved in C/M (1:1 v/v) to allow an adequate concentration for DE MALDI-TOF MS analysis.

Thin-layer chromatography

Lipid samples were applied to Silica gel 60-precoated plates and developed with C/M/W (65:25:4 v/v/v). The lipid bands were visualized with cupric sulfate pentahydrate phosphoric acid reagent at 150°C for 15 min (16).

DE MALDI-TOF MS

As previously described (7), 1 μl of a sample solution in C/M (1:1 v/v) containing about 100–500 pmol of the sample and 1 μl of the matrix solution (10 mg of 2,5-dihydroxybenzoic acid in 1 ml of 20% ethanol in water) in a 0.25-ml Eppendorff tube were shaken vigorously on a vortex mixer and spun down on a microcentrifuge. Supernatant (1 μl) was loaded into a well in a 100-well sample plate. Mass spectra of the sample in positive or negative ion mode were taken by a Voyager Biospectrometry (PerSeptive Biosystems) of DE MALDI-TOF MS in a reflector mode with N_2 laser (337 nm; step:2,300–2,700). A two-point external calibration was performed with psychosine [$(M+H)^+$: m/z 462.34 as sphingosine, d18:1] and globopentaosylceramide [$(M+Na)^+$: m/z 1564.94 as ceramide, d18:1C24:0] in the positive ion mode and lyso-sulfatide [$(M-H)^-$: m/z 540.28 as d18:1] and GM1 [$(M-H)^-$: m/z 1,544.87 as d18:1C18:0] in the negative ion mode. Five-point Savitsky-Golay smoothing was always applied to the mass spectra. Individual molecular species of glycero- and sphingo-glycolipids showed peaks of molecular ions (M^+) and isotopic ions [$(M+1)^+$ and $(M+2)^+$] 1 and 2 mass units higher than the molecular ion in expanded mass spectra. The percentage of the relative abundances (counts) of M^+, $(M+1)^+$, and $(M+2)^+$ peaks were almost identical, with the percentage of their theoretical abundances calculated from the natural abundances of ^{12}C, ^{13}C, ^1H, ^{14}N, and ^{16}O of individual glycolipid species. However, as the isotopic ion $(M+2)^+$ of the monounsaturated molecular species overlaps the M^+ of its saturated molecular species, an exact abundance of the latter ion peak must be corrected by subtraction of an abundance of the former ion peak deduced from the theoretical abundance as described above. After the correction for the ^{13}C isotope effect, the ion abundances of all M^+ of individual molecular species of the same class of glycolipids, such as cerebroside, are assumed to reflect their proportions, but this procedure does not give exact quantities.

RESULTS

Characterization of the minor components of neutral glycolipids

The neutral glycolipid fractions on Silica gel 60-column chromatography were checked by TLC and identified by DE MALDI-TOF MS. As can be seen in **Fig. 1**, the frac-

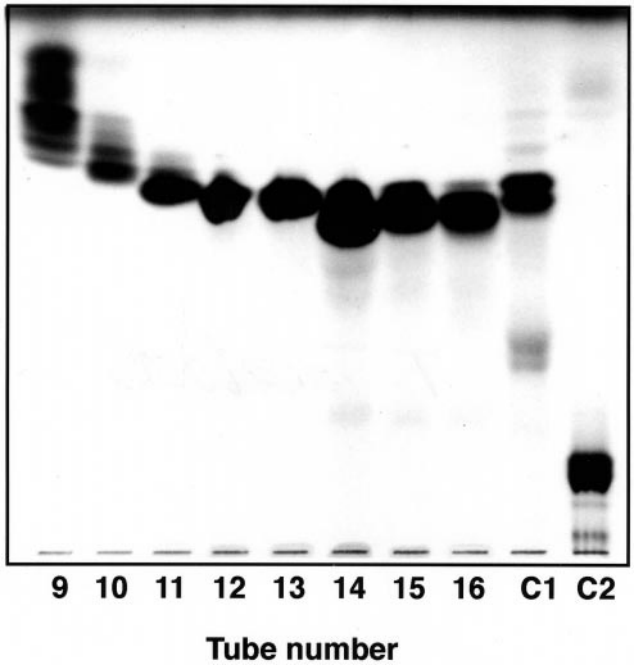

Fig. 1. TLC of the neutral glycolipid fractions eluted stepwise with chloroform-methanol (98:2 v/v), (95:5 v/v), (90:10 v/v), and (2:1 v/v) on a Silica gel 60-column chromatography, developed with chloroform-methanol-water (65:25:4 v/v/v) and visualized with cupric sulfate pentahydrate phosphoric acid reagent at 150°C for 15 min. Lane C1: authentic samples of cerebroside (upper) and sulfatide (lower). Lane C2: an authentic sample of sphingomyelin.

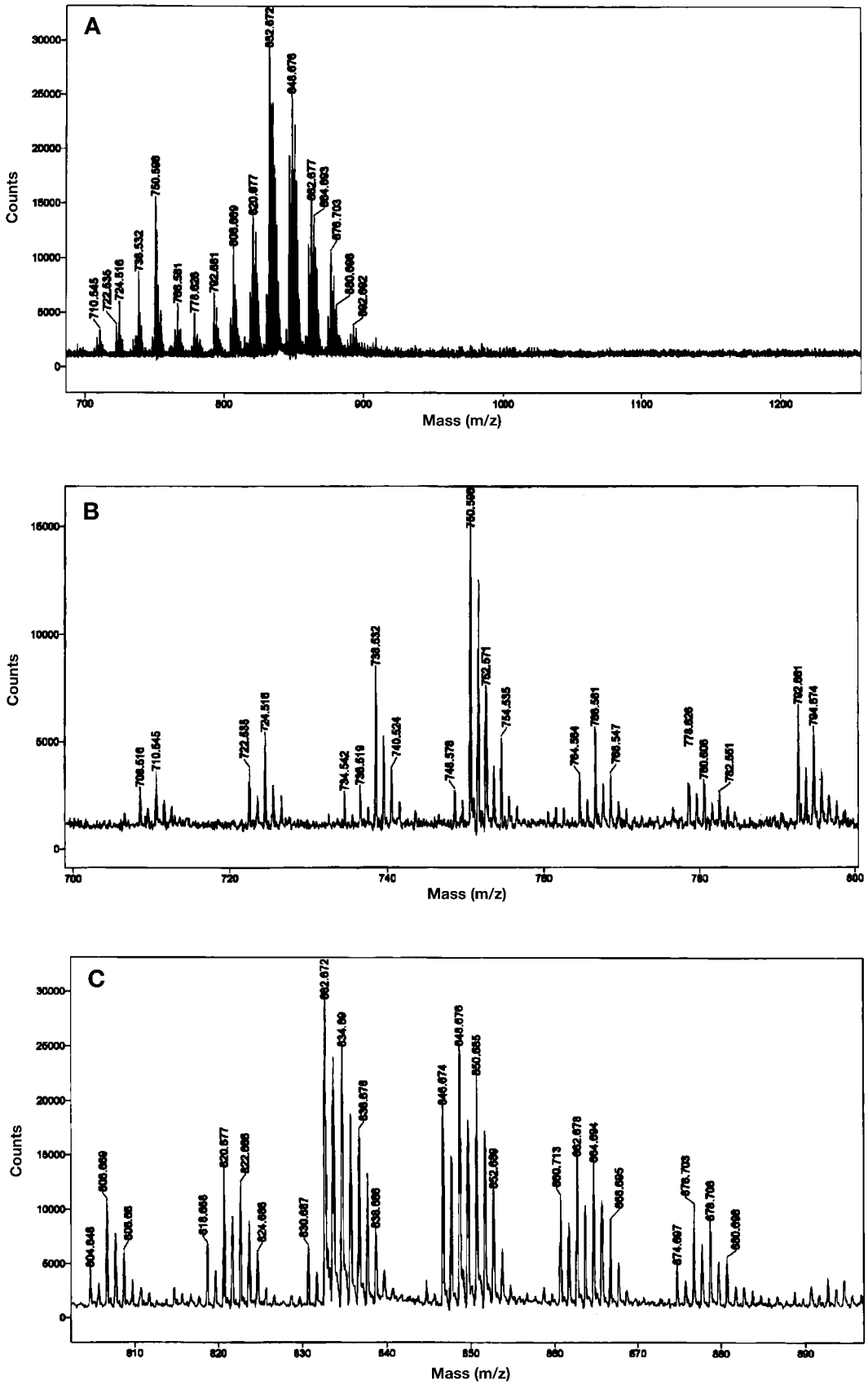

Fig. 2. A: DE MALDI-TOF mass spectra of cerebrosides in the combined fraction of tubes 12–16 at the range between m/z 700 and 1,200 measured with 2,5-DHB used as the matrix in the positive ion mode. B: Expanded mass spectra between m/z 700 and 800 in A. C: Expanded mass spectra between m/z 800 and 900 in A.

TABLE 1. Measured m/z of positive molecular ion signals assigned to individual molecular species of cerebrosides in the combined fraction of tubes 12–16

m/z	Molecular Ion	LCB	N-Fa	m/z	Molecular Ion	LCB	N-FA
708	$[M+Na]^+$	d18:1	C15:0	710	$[M+Na]^+$	d18:0	C15:0
722	$[M+Na]^+$	d18:1	C16:0	724	$[M+Na]^+$	d18:0	C16:0
734	$[M+Na]^+$	d18:1	C17:1	736	$[M+Na]^+$	d18:1	C17:0
738	$[M+Na]^+$	d18:1	C16:0h[a]	740	$[M+Na]^+$	d18:0	C16:0h
748	$[M+Na]^+$	d18:1	C18:1	750	$[M+Na]^+$	d18:1	C18:0
752	$[M+Na]^+$	d18:0	C18:0	754	$[M+Na]^+$	d18:1	C17:0h
764	$[M+Na]^+$	d18:1	C18:1h	766	$[M+Na]^+$	d18:1	C18:0h
768	$[M+Na]^+$	d18:0	C18:0h	778	$[M+Na]^+$	d18:1	C20:0
780	$[M+Na]^+$	d18:0	C20:0	782	$[M+H]^+$	d18:1	C22:1
792	$[M+Na]^+$	d18:1	C20:1h	794	$[M+Na]^+$	d18:1	C20:0h
804	$[M+Na]^+$	d18:1	C22:1	806	$[M+Na]^+$	d18:1	C22:0
808	$[M+Na]^+$	d18:0	C22:0	818	$[M+Na]^+$	d18:1	C23:1
820	$[M+Na]^+$	d18:1	C23:0	822	$[M+Na]^+$	d18:1	C22:0h
824	$[M+Na]^+$	d18:0	C22:0h	830	$[M+H]^+$	d18:0	C24:0h
832	$[M+Na]^+$	d18:1	C24:1	834	$[M+Na]^+$	d18:1	C24:0
836	$[M+Na]^+$	d18:1	C23:0h	838	$[M+Na]^+$	d18:0	C23:0h
846	$[M+Na]^+$	d18:1	C25:1	848	$[M+Na]^+$	d18:1	C24:1h
848	$[M+Na]^+$	d18:1	C25:0	850	$[M+Na]^+$	d18:1	C24:0h
852	$[M+Na]^+$	d18:0	C24:0h	860	$[M+Na]^+$	d18:1	C26:1
862	$[M+Na]^+$	d18:1	C25:1h	862	$[M+Na]^+$	d18:0	C26:0
864	$[M+Na]^+$	d18:1	C25:0h	866	$[M+Na]^+$	d18:0	C25:0h
874	$[M+Na]^+$	d18:1	C27:1	876	$[M+Na]^+$	d18:1	C26:1h
878	$[M+Na]^+$	d18:1	C26:0h	880	$[M+Na]^+$	d18:0	C26:0h

Abbreviation: N-FA, amide-linked fatty acid.
[a] h, hydroxy.

tions of tubes 9 and 10 showed the faster migrating elongated bands of less polar glycolipids than the cerebrosides showing bands in the fractions of tubes 12–16. First, an aliquot of the combined fraction of cerebrosides (tubes 12–16) was adequately concentrated and directly applied without any chemical treatment in advance of DE MALDI-TOF MS as described in Materials and Methods. As shown in **Fig. 2A**, the positive molecular ion signals of cerebrosides measured at the range between approximately m/z 700 and 1,200 were expanded between m/z 700 and 800 (Fig. 2B) and between m/z 800 and 900 (Fig. 2C), enough to assign the individual molecular species of cerebrosides as described in our previous paper (7). As shown in the figures, the molecular ion abundances of individual molecular species of cerebrosides (**Table 1**) indicated the characterization of mammalian brain cerebrosides containing major longer chain hydroxy and nonhydroxy fatty acids (17, 18).

Next, in comparison with the above cerebrosides, an aliquot of the combined fraction of less-polar glycolipids (tubes 9 and 10) was applied in the same manner to DE MALDI-TOF MS. As shown in **Fig. 3A**, the molecular ion signals measured between approximately m/z 680 and 1,200 were completely different from those of cerebrosides (Fig. 2A). The molecular ion signals between m/z 720 and 850 (Fig. 3B) in the expanded mass spectra corresponded

TABLE 2. Measured m/z of positive molecular ion signals assigned to individual molecular species of Galβ-1-Diradylglycerols (Galβ-1-diacyl-glycerols and Galβ-1-alkylacylglycerols) and ester cerebrosides in the less polar neutral glycolipids of the combined fraction of tubes 9 and 10

Galβ-1-Diradylglycerols

m/z	Molecular Ion	Diacyl	m/z	Molecular Ion	Alkylacyl
727	$[M+H]^+$	C14:0C18:2	727	$[M+H]^+$	C16:0C18:2
739	$[M+H]^+$	C15:0C18:3	751	$[M+H]^+$	C16:0C20:4
751	$[M+H]^+$	C14:0C20:4	753	$[M+H]^+$	C18:0C18:3
753	$[M+H]^+$	C16:0C18:3	765	$[M+H]^+$	C17:0C20:4
765	$[M+H]^+$	C15:0C20:4	779	$[M+H]^+$	C18:0C20:4
779	$[M+H]^+$	C16:0C20:4			
781	$[M+H]^+$	C18:0C18:3			
793	$[M+H]^+$	C17:0C20:4			
795	$[M+H]^+$	C19:0C18:3			
797	$[M+H]^+$	C19:0C18:2			
807	$[M+H]^+$	C18:0C20:4			
809	$[M+H]^+$	C20:0C18:3			
821	$[M+H]^+$	C19:0C20:4			
835	$[M+H]^+$	C20:0C20:4			

Ester Cerebrosides

m/z	Molecular Ion	LCB	N-FA	O-FA	m/z	Molecular Ion	LCB	N-FA	O-FA
1,071	$[M+Na]^+$	d18:1	C22:1	C18:0	1,073	$[M+Na]^+$	d18:1	C22:0	C18:0
1,085	$[M+Na]^+$	d18:1	C23:1	C18:0	1,087	$[M+Na]^+$	d18:1	C23:0	C18:0
1,089	$[M+Na]^+$	d18:1	C22:0h[a]	C18:0	1,099	$[M+Na]^+$	d18:1	C24:1	C18:0
1,101	$[M+Na]^+$	d18:1	C24:0	C18:0	1,103	$[M+Na]^+$	d18:1	C23:0h	C18:0
1,113	$[M+Na]^+$	d18:1	C25:1	C18:0	1,115	$[M+Na]^+$	d18:1	C24:1h	C18:0
1,115	$[M+Na]^+$	d18:1	C25:0	C18:0	1,117	$[M+Na]^+$	d18:1	C24:0h	C18:0
1,127	$[M+Na]^+$	d18:1	C26:1	C18:0	1,129	$[M+Na]^+$	d18:1	C25:1h	C18:0
1,129	$[M+Na]^+$	d18:1	C26:0	C18:0	1,131	$[M+Na]^+$	d18:1	C25:0h	C18:0
1,141	$[M+Na]^+$	d18:1	C27:1	C18:0	1,143	$[M+Na]^+$	d18:1	C27:0	C18:0

Abbreviations: O-FA, ester-linked fatty acid.
[a] h, hydroxy.

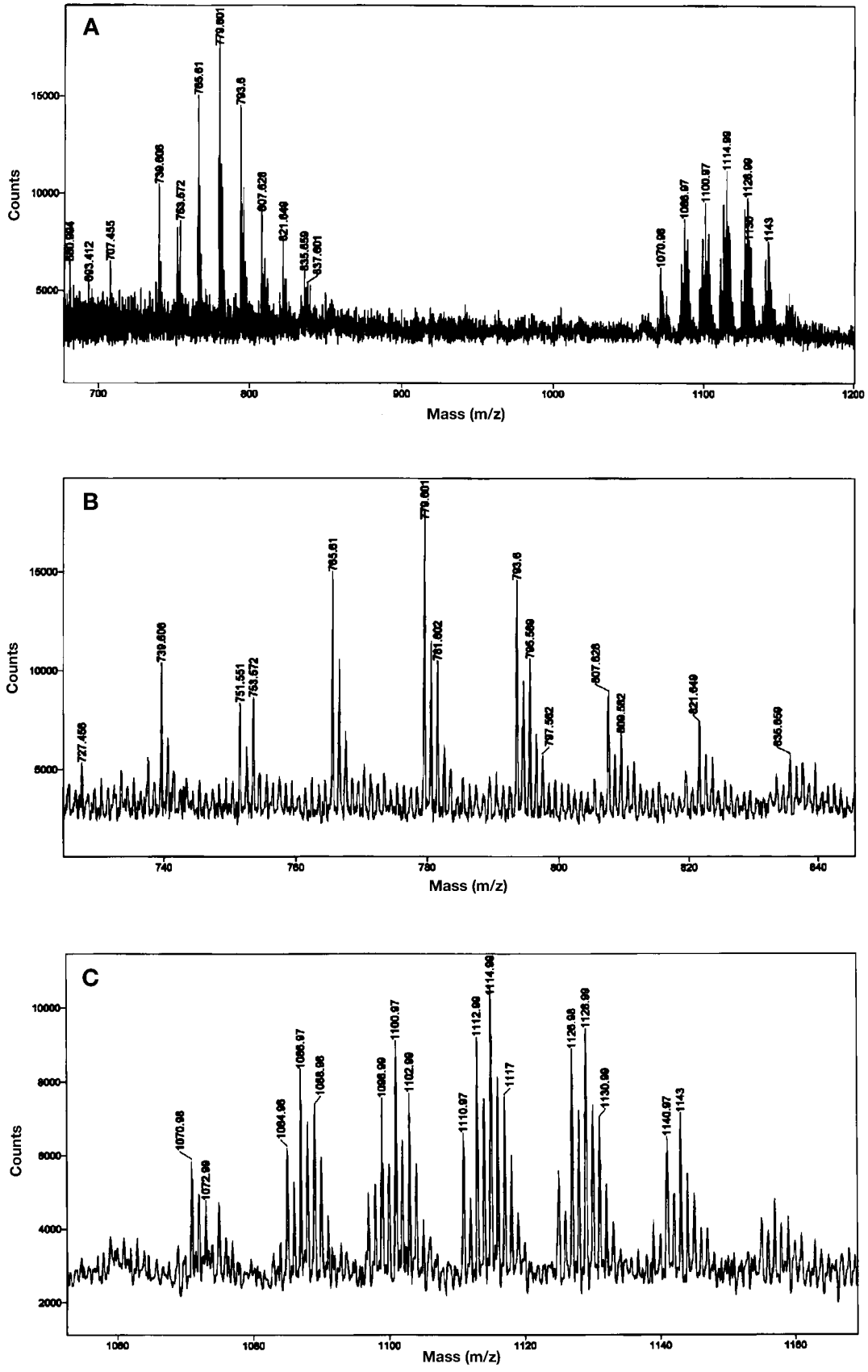

Fig. 3. A: DE MALDI-TOF mass spectra of less polar neutral glycolipids in the combined fraction of tubes 9 and 10 at the range between m/z 680 and 1,200 in the positive ion mode. B: Expanded mass spectra between m/z 720 and 850 in A. C: Expanded mass spectra between m/z 1,050 and 1,170 in A.

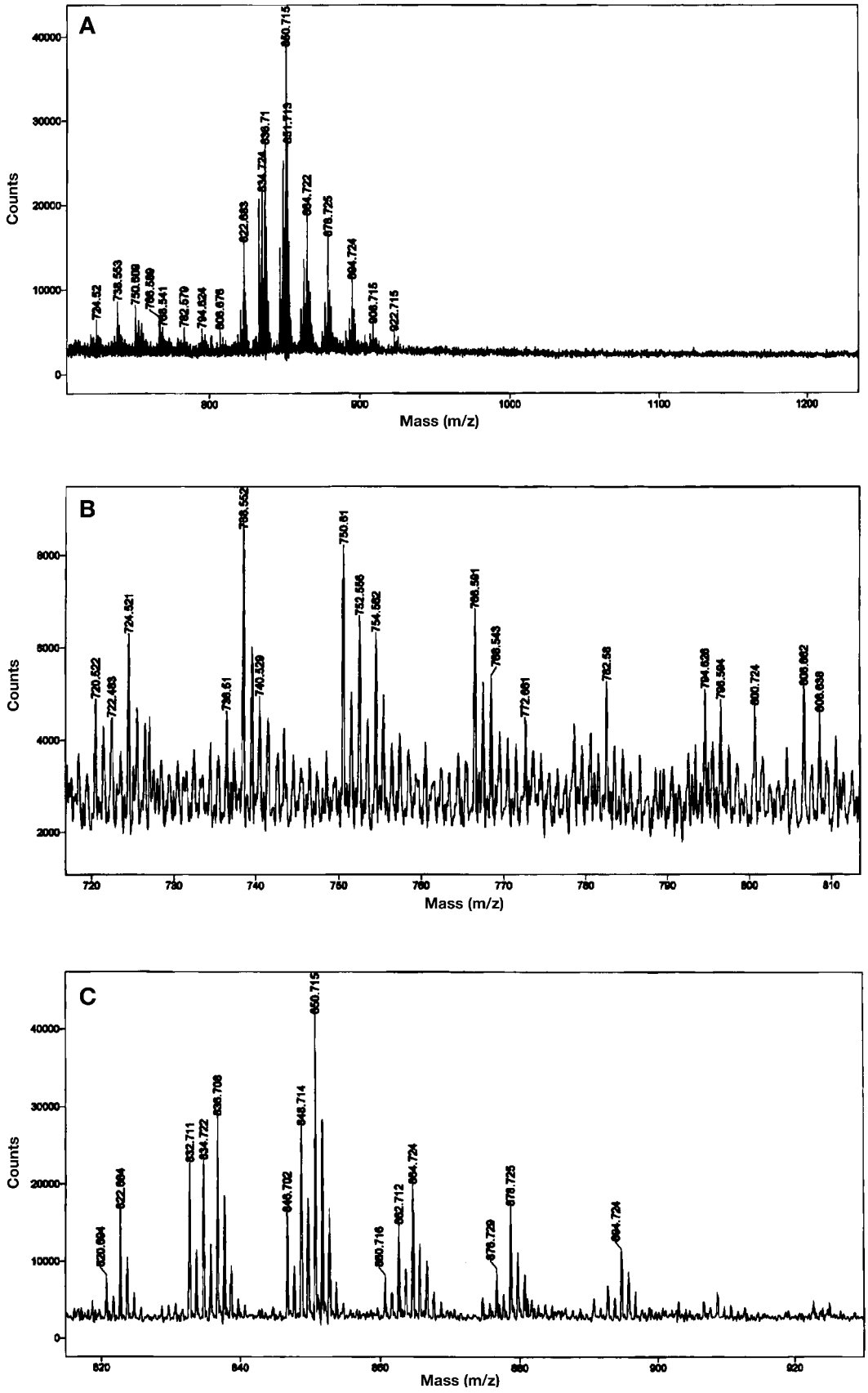

Fig. 4. A: DE MALDI-TOF mass spectra of the products derived from the less polar neutral glycolipids by mild alkaline hydrolysis at the range between m/z 710 and 1,200 in the positive ion mode. B: Expanded mass spectra between m/z 720 and 810 in A. C: Expanded mass spectra between m/z 820 and 920 in A.

to individual molecular species of Galβ-1-Diradylglycerols (mono-galactosyl-diacyl-glycerols and alkylacylglycerols) (12) in **Table 2**. The molecular ion abundances in Fig. 3B indicated that the Galβ-1-Diradylglycerols were composed mostly of Galβ-1-diacylglycerols and also Galβ-1-alkylacyl-glycerols, although their molecular ions at 727, 751, 753, 765, and 779 m/z overlapped each other and were not distinguished.

On the other hand, the ion signals between m/z 1,050 and 1,170 (Fig. 3C) corresponded to individual molecular species of ester cerebrosides or plasmalo-cerebrosides (12, 13), but only ester cerebrosides (Table 2) were identified by mild alkaline hydrolysis as follows: the products derived from the less polar neutral glycolipids by mild alkaline treatment showed the molecular ion signals between m/z 710 and 1,200 (Fig. 4A), which were completely different from the previous ones (Fig. 3A). Certainly, the mild alkali-labile Galβ-1-Diradylglycerols and ester cerebrosides were no longer found, but in place of them, only alkali-stable cerebrosides derived from ester cerebrosides were found between m/z 720 and 810 (Fig. 4B) and between m/z 820 and 920 (**Fig. 4C**), although a trace of alkali-stable plasmalo-cerebrosides seemed to be present in the less-polar neutral glycolipids of the brain tissues (5, 13, 14). The individual molecular species of cerebrosides derived from ester cerebrosides were also assigned in **Table 3** and resembled those of the cerebrosides in Table 1. Although the water-soluble mono-galactosyl glycerols and alkylglycerols derived from Galβ-1-Diradylglycerols by the mild alkaline hydrolysis are practically able to be analyzed by DE MALDI-TOF MS, the analysis of the water-soluble fraction was omitted in the present article. Thus, some molecular species of Galβ-1-Diradylglycerols still remain to be distinguished between mono-galactosyl diacylglycerols and alkylacylglycerols.

In conclusion, ester cerebrosides and Galβ-1-Diradylglycerols were confirmed by DE MALDI-TOF MS as minor components of neutral glycolipids in monkey brain tissue.

TABLE 3. Measured m/z of positive molecular ion signals assigned to individual molecular species of cerebrosides in the products derived from the less polar neutral glycolipids by mild alkaline hydrolysis

m/z	Molecular Ion	LCB	N-FA	m/z	Molecular Ion	LCB	N-FA
720	$[M+Na]^+$	d18:1	C16:1	722	$[M+Na]^+$	d18:1	C16:0
724	$[M+Na]^+$	d18:0	C16:0	736	$[M+Na]^+$	d18:1	C16:1h[a]
738	$[M+Na]^+$	d18:1	C16:0h	740	$[M+Na]^+$	d18:0	C16:0h
750	$[M+Na]^+$	d18:1	C18:0	752	$[M+Na]^+$	d18:0	C18:0
754	$[M+Na]^+$	d18:1	C20:1	766	$[M+Na]^+$	d18:1	C18:0h
768	$[M+Na]^+$	d18:0	C18:0h	772	$[M+H]^+$	d18:1	C20:0h
782	$[M+Na]^+$	d18:1	C22:1	794	$[M+Na]^+$	d18:1	C20:0h
796	$[M+Na]^+$	d18:0	C20:0h	800	$[M+Na]^+$	d18:1	C22:0h
806	$[M+Na]^+$	d18:1	C20:0	808	$[M+Na]^+$	d18:0	C22:0
820	$[M+Na]^+$	d18:1	C23:0	822	$[M+Na]^+$	d18:1	C23:0
832	$[M+Na]^+$	d18:1	C24:1	834	$[M+Na]^+$	d18:1	C24:0
836	$[M+Na]^+$	d18:1	C23:0h	846	$[M+Na]^+$	d18:1	C25:1
848	$[M+Na]^+$	d18:1	C24:1h	850	$[M+Na]^+$	d18:1	C24:0h
860	$[M+Na]^+$	d18:1	C26:1	862	$[M+Na]^+$	d18:1	C26:0
864	$[M+Na]^+$	d18:1	C25:0h	876	$[M+Na]^+$	d18:1	C27:0
878	$[M+Na]^+$	d18:1	C26:0h	894	$[M+Na]^+$	d18:1	C27:0h

[a] h, hydroxy.

Characterization of the minor components of acidic glycolipids

The acidic lipids separated from the above neutral lipids by a DEAE-Sephadex A-25 (acetate form) were fractionated with Silica gel 60-column chromatography with linear gradient of C/M/W (80:20:0.5 v/v/v) to C/M/W (10:90:5 v/v/v). The different fractions were checked by TLC, and, as shown in **Fig. 5**, the fraction of tube 5 showed faster migrating of several bands of less polar acidic glycolipids than sulfatides showing bands in the fractions of tubes 8–12. The fractions of tubes 6 and 7 showing both the faster migrating bands and sulfatide bands were separated into the less polar acidic glycolipid and sulfatide portions by a preparative high-performance TLC. The faster migrating less polar acidic glycolipids were directly applied to DE MALDI-TOF MS in a negative ion mode. As shown in **Fig. 6A**, the negative molecular ion signals measured between m/z 860 and 1,250 gave much larger molecular weights than those of the usual sulfatides. They were expanded as shown in Fig. 6B and assigned as individual molecular species of C18:0-ester sulfatides (15) and novel C18:0-aldehyde plasmalo-sulfatides, C18:0-ester HSO_3-Galβ-1-Diradylglycerols, and C-18:0-aldehyde plasmalo HSO_3-Galβ-1-Diradylglycerols (**Table 4**). The ester sulfatides and plasmalo-sulfatides were clearly distinguished by mild alkaline hydrolysis, whereas intact ester HSO_3-

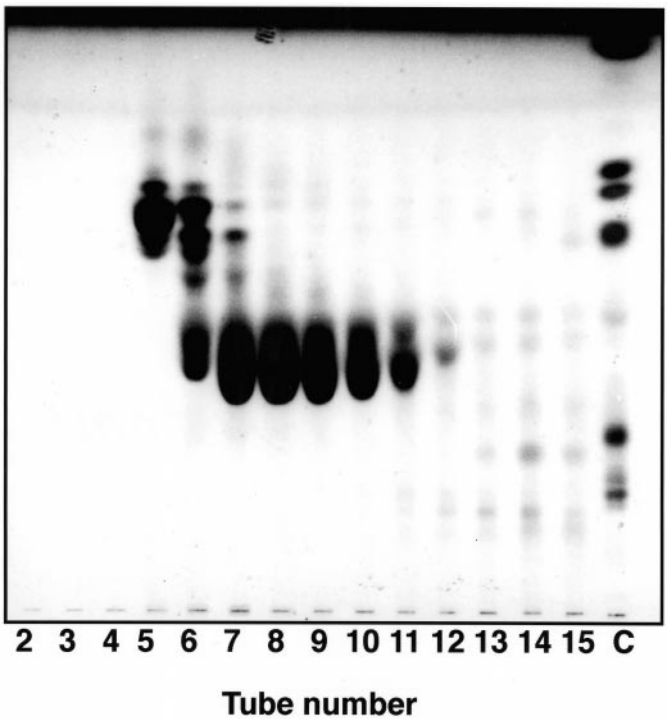

Fig. 5. TLC of the acidic glycolipid fractions eluted with linear gradient of chloroform-methanol-water (30:60:8 v/v/v) to (10:90:5 v/v/v) on a Silica gel 60-column chromatography. Developed with chloroform/methanol/water (60:25:4 v/v/v) and visualized in the same manner as in Fig. 1. Lane C: Authentic samples of cholesterol, cerebrosides (double bands), phosphatidylethanolamine, sulfatide (faint band), phosphatidylcholine, and sphingomyelin, in the order from the top.

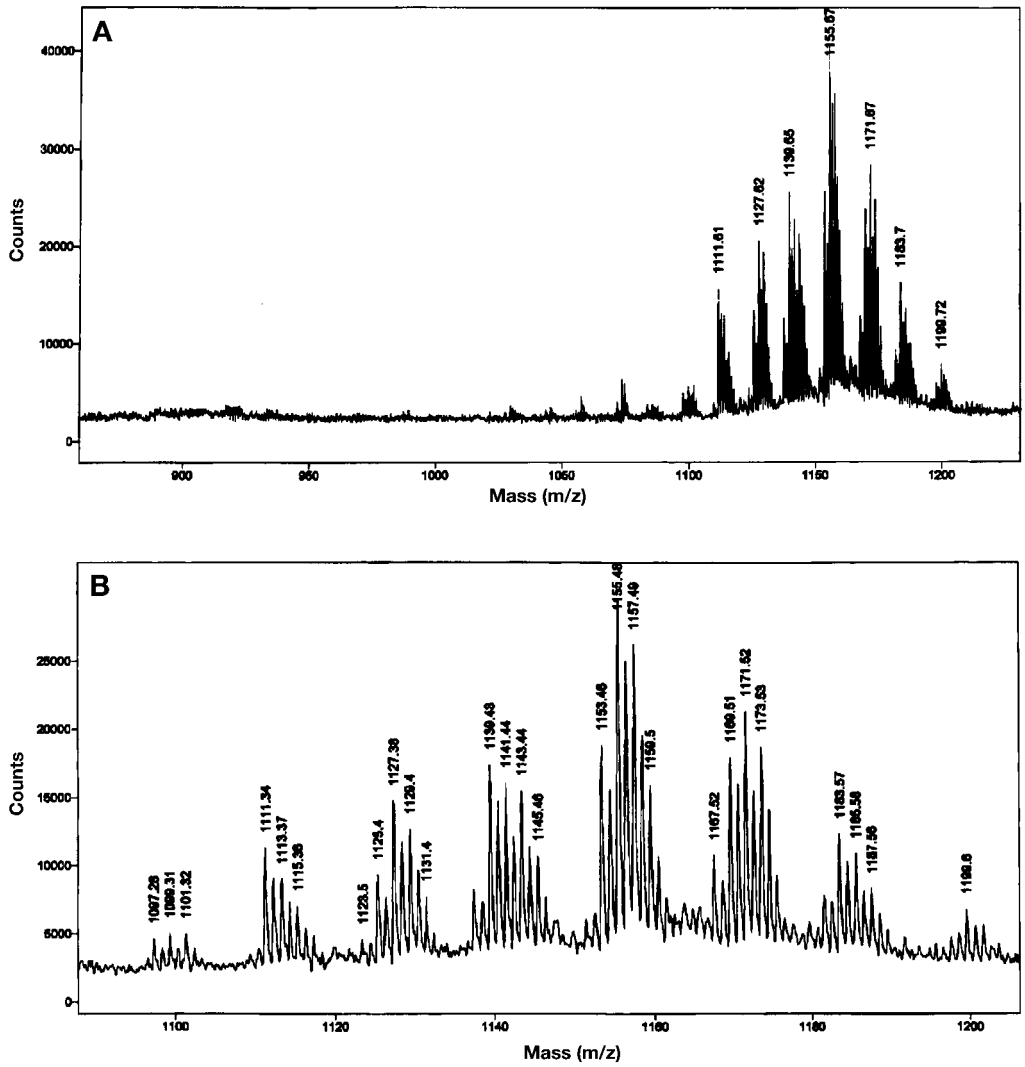

Fig. 6. A: DE MALDI-TOF mass spectra of less polar acidic glycolipid fraction at the range between m/z 860 and 1,250 measured with 2,5-DHB used as the matrix in the negative ion mode. B Expanded mass spectra between m/z 1,090 and 1,210 in A.

3Galβ-1-Diradylglycerols (diacyl- and alkylacyl-glycerols) and plasmalo HSO_3-3Galβ-1-Diradylglycerols were able to be distinguished but were found to overlap some molecular species of ester sulfatides and plasmalo-sulfatides. Thus, the intact and mild alkali-treated less polar acidic glycolipids were compared with each other as described below.

Next, an aliquot of the combined sulfatide fraction was directly applied to DE MALDI-TOF MS. As shown in **Fig. 7A**, the negative molecular ion signals of the fraction measured between m/z 740 and 1,250 were clearly different from those of the less polar acidic glycolipids (Fig. 6A). The molecular ion signals between m/z 740 and 850 were expanded as shown in Fig. 7B, and the molecular ion signals between m/z 870 and 970 were expanded as in Fig. 7C. As shown in **Table 5**, they were assigned as individual molecular species of sulfatides and HSO_3-3Galβ-1-Diradylglycerols. The latter consisted of both diacyl and alkylacyl groups and overlapped some molecular species of sulfatides. However, the products derived from the combined sulfatide fraction by mild alkaline hydrolysis showed molecular ion signals of only sulfatides (data not shown), which were slightly different from the previous molecular ion signals (Fig. 7), indicating the presence of trace amounts of HSO_3-3Galβ-1-Diradylglycerols that were lost by the alkaline treatment.

Also, the products derived from the less polar acidic glycolipids by mild alkaline hydrolysis showed the negative molecular ion signals measured between m/z 750 and 1,250 (**Fig. 8A**), which were completely different from the previous mass spectra (Fig. 6A) and showed a major group between m/z 840 and 950 and a minor group between approximately m/z 1,100 and 1,200. The former was expanded as shown in Fig. 8B and assigned as individual molecular species of sulfatides (**Table 6**), derived from ester sulfatides that were found to be a major portion of the less polar acidic glycolipids, whereas the latter was expanded as in Fig. 8C and as already described in Table 4, assigned as molecular species of alkali-stable plasmalo-sulfatides that were found in trace amounts. The molecular ion signals of alkali-labile ester HSO_3-3Galβ-1-Diradylglycerols

TABLE 4. Measured m/z of the negative molecular ion signals assigned to individual molecular species of ester sulfatides, plasmalo-sulfatides, ester HSO_3-3Galβ-1-Diradylglycerols, and plasmalo HSO_3-3Galβ-1-Diradyl-glycerols in the less polar acidic glycolipid fraction

Ester Sulfatides

m/z	Molecular Ion	LCB	N-FA	O-FA	m/z	Molecular Ion	LCB	N-FA	O-FA
1,129	$[M-H]^-$	d18:1	C22:0	C18:0	1,143	$[M-H]^-$	d18:1	C23:0	C18:0
1,155	$[M-H]^-$	d18:1	C24:1	C18:0	1,157	$[M-H]^-$	d18:1	C24:0	C18:0
1,159	$[M-H]^-$	d18:1	C23:0h[a]	C18:0	1,169	$[M-H]^-$	d18:1	C25:1	C18:0
1,171	$[M-H]^-$	d18:1	C24:1h	C18:0	1,171	$[M-H]^-$	d18:1	C25:0	C18:0
1,173	$[M-H]^-$	d18:1	C24:0h	C18:0	1,183	$[M-H]^-$	d18:1	C26:1	C18:0
1,185	$[M-H]^-$	d18:1	C26:0	C18:0	1,187	$[M-H]^-$	d18:1	C25:0h	C18:0
1,199	$[M-H]^-$	d18:1	C27:0	C18:0					

Plasmalo-sulfatides

m/z	Molecular Ion	LCB	N-FA	Aldehyde	m/z	Molecular Ion	LCB	N-FA	Aldehyde
1,111	$[M-H]^-$	d18:1	C22:1	C18:0	1,113	$[M-H]^-$	d18:1	C22:0	C18:0
1,125	$[M-H]^-$	d18:1	C23:1	C18:0	1,127	$[M-H]^-$	d18:1	C23:0	C18:0
1,139	$[M-H]^-$	d18:1	C24:1	C18:0	1,141	$[M-H]^-$	d18:1	C24:0	C18:0
1,153	$[M-H]^-$	d18:1	C25:1	C18:0	1,155	$[M-H]^-$	d18:1	C24:1h	C18:0
1,155	$[M-H]^-$	d18:1	C25:0	C18:0					

Ester HSO_3-3Galβ-1-Diradylglycerols

m/z	Molecular Ion	Diacyl	O-FA	m/z	Molecular Ion	Alkylacyl	O-FA
1,097	$[M-H]^-$	C16:0C18:3	C18:0	1,099	$[M-H]^-$	C18:0C18:2	C18:0
1,099	$[M-H]^-$	C16:0C18:2	C18:0	1,101	$[M-H]^-$	C18:0C18:1	C18:0
1,101	$[M-H]^-$	C16:0C18:1	C18:0				
1,123	$[M-H]^-$	C16:0C20:4	C18:0				
1,125[b]	$[M-H]^-$	C18:0C18:3	C18:0				
1,127[b]	$[M-H]^-$	C18:0C18:2	C18:0				
1,129[c]	$[M-H]^-$	C18:0C18:1	C18:0				
1,131	$[M-H]^-$	C18:0C18:0	C18:0				

Plasmalo HSO_3-3Galβ-1-Diradylglycerols

m/z	Molecular Ion	Diacyl	Aldehyde	m/z	Molecular Ion	Alkylacyl	Aldehyde
1,111[b]	$[M-H]^-$	C18:1C18:2	C18:0	1,153[b]	$[M-H]^-$	C18:1C18:3	C18:0
1,113[b]	$[M-H]^-$	C18:1C18:1	C18:0	1,155[b]	$[M-H]^-$	C18:1C18:2	C18:0
1,115	$[M-H]^-$	C18:1C18:0	C18:0	1,157[c]	$[M-H]^-$	C18:1C18:1	C18:0
1,141[b]	$[M-H]^-$	C18:1C20:1	C18:0	1,159[c]	$[M-H]^-$	C18:1C18:0	C18:0
1,143[c]	$[M-H]^-$	C18:1C20:0	C18:0				

[a] h, hydroxy.
[b] Overlapped plasmalo-sulfatide.
[c] Overlapped ester sulfatide.

and plasmalo HSO_3-3Galβ-1-Diradylglycerols, some of which overlapped the ester sulfatides and plasmalo-sulfatides, were no longer observed in Fig. 8, but, in consideration of the difference between the mass spectra in Figs. 6 and 8, we concluded that the less polar acidic glycolipids contained, in order of amount, ester sulfatides > plasmalo HSO_3-3Galβ-1-Diradylglycerols, ester HSO_3-3Galβ-1-Diradylglycerols > plasmalo-sulfatides. The combined sulfatide fraction was also concluded to contain trace amounts of HSO_3-3Galβ-1-Diradylglycerols.

DISCUSSION

We have recently used DE MALDI-TOF MS, improved with the new delayed ion extraction technique, to analyze sphingoglycolipids and related materials of which the chemical structures had been known (1–7). Certainly, this technique, which offers the advantages of sensitivity, mass accuracy, and simplicity (7), is expected for the rapid confirmation and identification of numerous sphingoglycolipide that, beginning with the discovery of brain cerebroside by Thudichum (19), have been revealed particularly during the decades after World War II.

In this study, the minor components of neutral and acidic glycolipids, which consist of various kinds of glycero- and sphingo-glycolipids with their peculiar chemical structures, have been clarified by DE MALDI-TOF MS analysis without any special chemical treatment, except mild alkaline hydrolysis.

Unexpectedly, in addition to the confirmation of already-known ester cerebrosides, Galβ-1-Diradylglycerols, ester

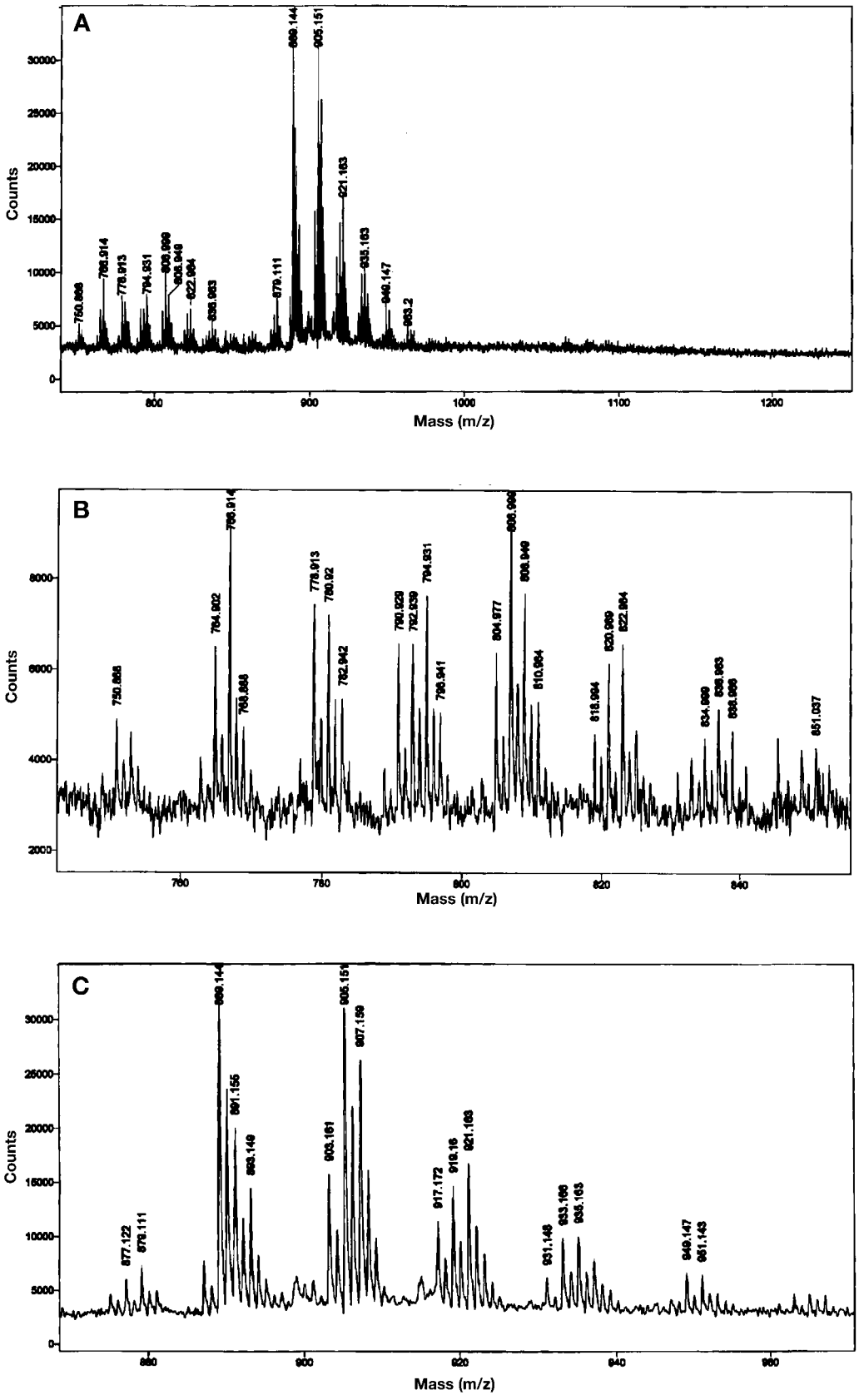

Fig. 7. A: DE MALDI-TOF mass spectra of the combined sulfatide fraction at the range between m/z 740 and 1,250 in the negative ion mode. B: Expanded mass spectra between m/z 740 and 850 in A. C: Expanded mass spectra between m/z 870 and 970 in A.

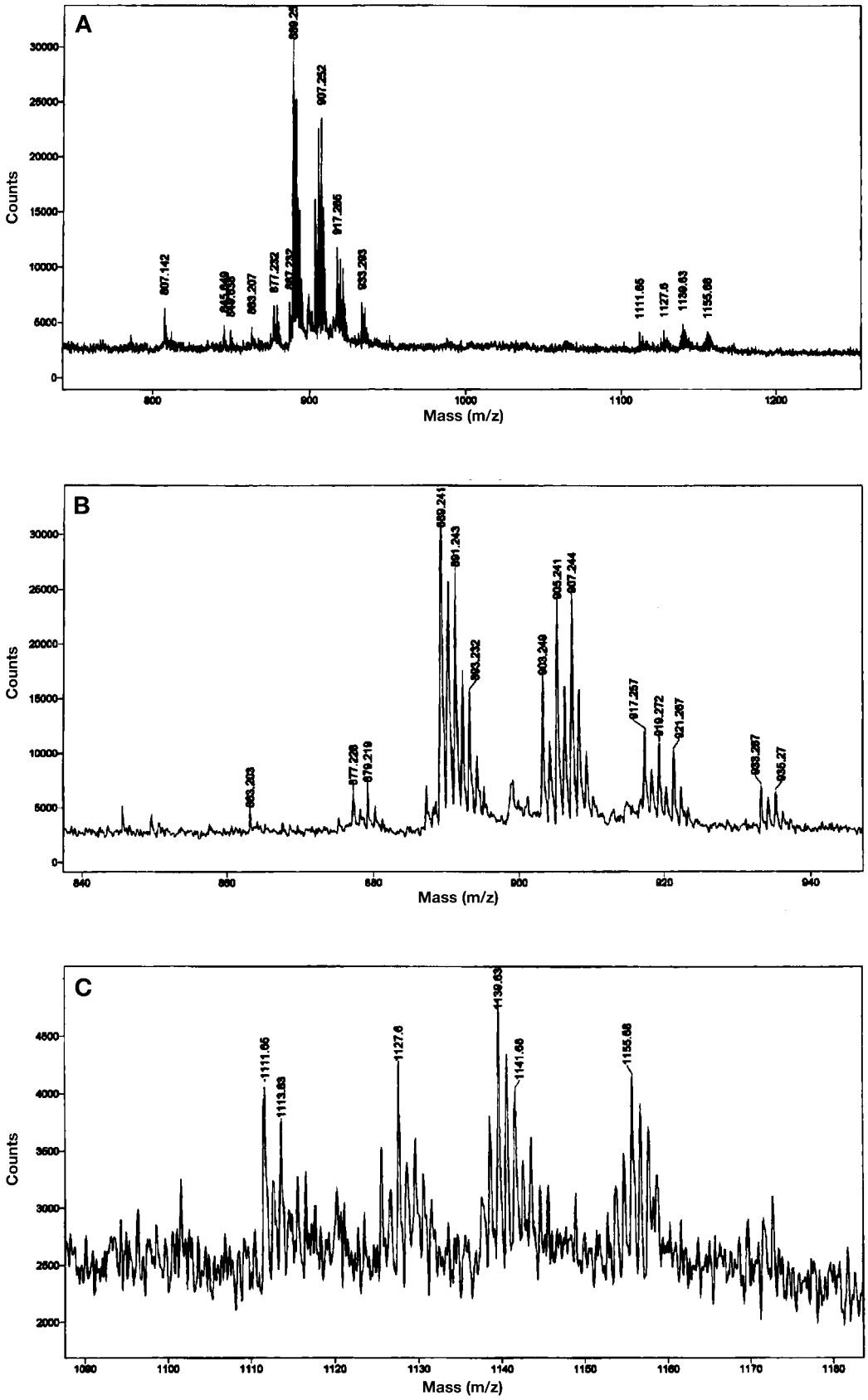

Fig. 8. A: DE MALDI-TOF mass spectra of the products derived from the less polar acidic glycolipids by mild alkaline hydrolysis at the range between m/z 750 and 1,250 in the negative ion mode. B: Expanded mass spectra between m/z 840 and 950 in A. C: Expanded mass spectra between m/z 1,090 and 1,200 in A.

TABLE 5. Measured m/z of the negative molecular ion signals assigned to individual molecular species of sulfatides and HSO_3-3Galβ-1-Diradylglycerols in the combined sulfatide fraction

Sulfatides

m/z	Molecular Ion	LCB	N-FA	m/z	Molecular Ion	LCB	N-FA
779	$[M-H]^-$	d18:1	C16:0	781	$[M-H]^-$	d18:0	C16:0
797	$[M-H]^-$	d18:0	C16:0h[a]	805	$[M-H]^-$	d18:1	C18:1
807	$[M-H]^-$	d18:1	C18:0	809	$[M-H]^-$	d18:0	C18:0
823	$[M-H]^-$	d18:1	C18:0h	835	$[M-H]^-$	d18:1	C20:1
837	$[M-H]^-$	d18:1	C20:0	851	$[M-H]^-$	d18:1	C20:0h
877	$[M-H]^-$	d18:1	C23:0	879	$[M-H]^-$	d18:1	C22:0h
889	$[M-H]^-$	d18:1	C24:1	891	$[M-H]^-$	d18:1	C24:0
893	$[M-H]^-$	d18:1	C23:0h	903	$[M-H]^-$	d18:1	C25:1
905	$[M-H]^-$	d18:1	C24:1h	905	$[M-H]^-$	d18:1	C25:0
907	$[M-H]^-$	d18:1	C24:0h	917	$[M-H]^-$	d18:1	C26:1
919	$[M-H]^-$	d18:1	C25:1h	919	$[M-H]^-$	d18:1	C26:0
921	$[M-H]^-$	d18:1	C25:0h	931	$[M-H]^-$	d18:1	C27:1
933	$[M-H]^-$	d18:1	C27:0	933	$[M-H]^-$	d18:1	C26:1h
935	$[M-H]^-$	d18:1	C26:0h	949	$[M-H]^-$	d18:1	C27:1h
951	$[M-H]^-$	d18:0	C27:0h				

HSO_3-3Galβ-1-Diradylglycerols

m/z	Molecular Ion	Diacyl	Alkylacyl
751	$[M-H]^-$	C12:0C16:1	C14:0C16:1
765	$[M-H]^-$	C13:0C16:1	C15:0C16:1
779[b]	$[M-H]^-$	C14:0C16:1	C16:0C16:1
781[b]	$[M-H]^-$	C14:0C16:0	C16:0C16:0
805[b]	$[M-H]^-$	C14:0C18:2	C16:0C18:2
807[b]	$[M-H]^-$	C16:0C16:1	C16:0C18:1
809[b]	$[M-H]^-$	C16:0C16:0	C16:0C18:0
833	$[M-H]^-$	C16:0C18:2	C18:0C18:2
835[b]	$[M-H]^-$	C16:0C18:1	C18:0C18:1
837[b]	$[M-H]^-$	C16:0C18:0	C18:0C18:0

[a] h, hydroxy.
[b] Overlapped sulfatides.

TABLE 6. Measured m/z of the negative molecular ion signals assigned to individual molecular species of sulfatides and plasmalo-sulfatides in the products derived from the less polar acidic glycolipids by mild alkaline hydrolysis

m/z	Molecular Ion	LCB	N-FA	m/z	Molecular Ion	LCB	N-FA
863	$[M-H]^-$	d18:1	C22:0	877	$[M-H]^-$	d18:1	C23:0
879	$[M-H]^-$	d18:1	C22:0h[a]	889	$[M-H]^-$	d18:1	C24:1
891	$[M-H]^-$	d18:1	C24:0	893	$[M-H]^-$	d18:1	C23:0h
903	$[M-H]^-$	d18:1	C25:1	905	$[M-H]^-$	d18:1	C24:1h
905	$[M-H]^-$	d18:1	C25:0	907	$[M-H]^-$	d18:1	C24:0h
917	$[M-H]^-$	d18:1	C26:1	919	$[M-H]^-$	d18:1	C25:1h
919	$[M-H]^-$	d18:1	C26:0	921	$[M-H]^-$	d18:1	C25:0h
933	$[M-H]^-$	d18:1	C27:0	935	$[M-H]^-$	d18:1	C26:0h

[a] h, hydroxy.

sulfatides, and HSO_3-3Galβ-1-Diradylglycerols (8–15), novel C18-ester HSO_3-Galβ-1-Diradylglycerols, C18-aldehyde plasmalo HSO_3-Galβ-1-Diradylglycerols, and C18-aldehyde plasmalo-sulfatides have been found to be minor components of acidic glycolipids in monkey brain tissue, but plasmalo-cerebrosides (5, 13, 14) were not found. The diacyl and alkylacyl groups of Galβ-1-Diradylglycerols and HSO_3-3Galβ-1-Diradylglycerols still remain to be distinguished, but if the water-soluble fraction of galactosylglycerol, galactosylalkylglycerol, HSO_3-3Galβ-1-glycerol, and HSO_3-3Galβ-1-alkylglycerol derived from the Diradylglycerols by mild alkaline hydrolysis are identified by DE MALDI-TOF MS analysis, the galactosyl or sulfo-galactosyl diacylglycerols and alkylacylglycerols probably can be distinguished.

It may be emphasized that the information of positive or negative molecular ions of minute amounts of the samples by DE MALDI-TOF MS analysis in a positive or negative ion mode is helpful to distinguish between neutral and acidic glycolipids such as cerebroside and sulfatide, and that intact minor components of neutral and acidic glycolipids, in which the molecular weights are very similar to each other, can be distinguished with the accurate mass-to-charge ratios measured by DE MALDI-TOF MS analysis. Needles to say, the individual molecular ion signals highly resolved in DE MALDI-TOF mass spectra will be able to be easily and rapidly assigned by deduction from the molecular weights with more experimental knowledge and experiences on various kinds of glycolipids.

Although this analysis of the mass spectrum after the correction for ^{13}C isotope effects provides a quantitative analysis of individual molecular species of at least the same class of glycolipids, such as cerebroside or sulfatide, this procedure does not give exact quantities (7).

Rapid and accurate analysis of minute amounts of glycolipids by the simple and sensitive method of DE MALDI-TOF MS instead of the general radio-isotopic technique may be necessary for the clarification of the unknown distribution, function, and metabolism of minor components of neutral and acidic glycolipids in brain tissue in the near future.

This research is dedicated to J. Ludwig W. Thudichum, M.D., the father of brain chemistry, in commemoration of the centennial of his death in 2001.

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REFERENCES

1. Taketomi, T., A. Hara, K. Uemura, and E. Sugiyama. 1996. Rapid method of preparation of lysoglycosphingolipids and their confirmation by delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *J. Biochem.* **120:** 573–579.
2. Taketomi, T., A. Hara, K. Uemura, H. Kurahashi, and E. Sugiyama. 1996. A microwave-mediated saponification of galactosylceramide and galacto-sylceramide I^3-sulfate and identification of their lyso-compounds by delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Biochem. Biophys. Res. Commun.* **224:** 462–467.
3. Taketomi, T., A. Hara, K. Uemura, H. Kurahashi, and E. Sugiyama. 1997. Preparation of various lysogangliosides including lyso-fucosyl GM1 and delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometric analysis. *J. Biochem.* **121:** 264–269.
4. Sugiyama, E., A. Hara, K. Uemura, and T. Taketomi. 1997. Application of matrix-assisted laser desorption ionization time-of-flight mass spectrometry with delayed ion extraction to ganglioside analysis. *Glycobiology.* **7:** 719–724.
5. Taketomi, T., A. Hara, K. Uemura, and E. Sugiyama. 1998. Matrix-

assisted laser desorption ionization time-of-flight mass spectrometric analysis of glycosphingolipids including gangliosides. *Acta Biochim. Pol.* **45:** 987–999.
6. Fujiwaki, T., S. Yamaguchi, K. Sukegawa, and T. Taketomi. 1999. Application of delayed extraction matrix-assisted laser desorption ionization time-of-flight mass spectrometry for analysis of sphingolipids in tissues from sphingolipidosis patients. *J. Chromatogr. B. Biomed. Appl.* **731:** 45–52.
7. Taketomi, T., and E. Sugiyama. 2000. Extraction and analysis of multiple sphingolipids from a simple sample. *Methods Enzymol.* **312:** 80–101.
8. Klenk, E., and M. Doss. 1966. Über das Vorkommen von Estercerebrosiden in Gehirn. *Z. Physiol. Chem.* **346:** 296–298.
9. Tamai, Y. 1968. Further study on the faster running glycolipid in brain. *Jpn. J. Exp. Med.* **38:** 65–73.
10. Kishimoto, Y., M. Wajda, and N. S. Radin. 1968. 6'-Acyl galactosyl ceramides of pig brain: structure and fatty acid composition. *J. Lipid Res.* **9:** 27–33.
11. Yasugi, E., T. Kasama, H. Kojima, and T. Yamakawa. 1983. Occurrence of 2-O-acyl galactosylceramide in human and bovine brains. *J. Biochem.* **93:** 1595–1599.
12. Shimomura, K., and Y. Kishimoto. 1984. Changes in monogalactosyl diacylglycerol, alkylgalactolipids and cerebroside fatty acid esters in maturing rat brain measured by high-performance liquid chromatography. *Biochim. Biophys. Acta.* **794:** 162–164.
13. Levery, S. B., E. D. Nudelman, and S. Hakomori. 1992. Novel modification of glycosphingolipids by long-chain cyclic acetals: Isolataion and characterization of plasmalocerebroside from human brain. *Biochemistry.* **31:** 5335–5340.
14. Yachida, Y., M. Kashiwagi, T. Mikami, K. Tsuchihashi, T. Daino, T. Akino, and S. Gasa. 1998. Stereochemical structures of synthesized and natural plasmalogalactosylceramides from equine brain. *J. Lipid Res.* **39:** 1039–1045.
15. Mikami, T., K. Tsuchihashi, M. Kashiwagi, Y. Yachida, T. Daino, K. Hashi, T. Akino, and S. Gasa.1998. Characterization of a O-fatty-acylated sulfatide from equine brain. *Eur. J. Biochem.* **255:** 289–295.
16. Hara, A., and T. Taketomi. 1991. Characterization and changes of glycosphingolipids in the aorta of the Watanabe hereditary rabbit. *J. Biochem.* **109:** 904–908.
17. Hara, A., N. Kitazawa, and T. Taketomi. 1984. Abnormalities of glyco-sphingolipids in mucopolysaccharidosis Type IIIB. *J. Lipid Res.* **25:** 175–184.
18. Sato, S., N. Kawamura, and T. Taketomi. 1979. Brain glycolipids of a wild Japanese adult bear. *Jpn. J. Exp. Med.* **49:** 139–146.
19. Thudichum, J. L. W. 1884. A Treatise on the Chemical Constitution of the Brain. Ballière, Tindall, and Cox, London.