[8] Extraction and Analysis of Multiple Sphingolipids from a Single Sample

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Introduction

A long and fascinating history of sphingolipid research, beginning with the discovery of brain sphingomyelin and cerebroside containing sphingosine as the characteristic unit of the sphingolipids in 1884 by Thudichum,[1] has established the chemical structures of numerous sphingolipids in animal tissues and cells. Particularly during the decades after World War II, improved methods of isolation and structure determination have revealed an astonishing variety of oligosaccharide structures that occur in the 300 or more glycosphingolipids.[2] Biologic functions of trace amounts of exogenous and endogenous sphingolipids in many vertebral and invertebral cells now command a great deal of attention. The chemical identification of the sphingolipids and related materials in the cells or tissues always should be prerequisite to studies of their function and undertaken easily, quickly, and accurately.

Fortunately, an excellent method, matrix-assisted laser desorption ionization time-of-fight mass spectrometry with delayed ion extraction (DE MALDI-TOF MS), can be applied to the identification and analysis of sphingolipids extracted from individual animal cells or tissues. In this chapter we describe the application of DE MALDI-TOF MS to gala-, globo-, and ganglio-series glycosphingolipids and sphingomyelin as well as some examples of experimental procedures and practical methods for simultaneous analysis of multiple sphingolipids in individual animal cells or tissues.

Principle of DE MALDI-TOF MS

In matrix-assisted laser desorption ionization (MALDI), the sample is embedded in a low molecular weight, UV-absorbing matrix [2,5-dihydroxybenzoic acid (2,5-DHB) or α-4-hydroxycinnamic acid (α-4-CHCA)] that

[1] J. L. W. Thudichum, "A Treatise on the Chemical Constitution of the Brain." Ballière, Tindall & Cox, London, 1884.

[2] R. W. Ledeen, *in* "Sphingolipids as Signaling Modulators in the Nervous System" (R. W. Ledeen, S. Hakomori, A. J. Yates, J. S. Schneider, and R. K. Yu, eds.), *Ann. N.Y. Acad. Sci.* **845,** xi (1998).

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enhances sample ionization. While the exact role of the matrix is not completely defined, it appears to transfer enough energy to the sample to ionize it to a molecular ion, but not enough energy to fragment the ions into atomic components.

Time-of-flight mass spectrometry works on the principle that if ions are accelerated with the same kinetic energy from a fixed point and at a fixed initial time, the ions will separate according to their mass/charge ratios. Light ions travel more quickly to the analyzer, heavier ions more slowly. The time required for ions to reach the detector and the ion intensity (abundance) are measured. Drift time is proportional to the square root of the mass:

$$t = s(m/2KE)^{1/2}$$

where t is the drift time, s is the drift distance, m is mass, and $EK = 1/2m(s/t)^2$.

Furthermore, MALDI-TOF MS with the delayed ion extraction (DE) technique has the advantages of a broad mass range (>100 kDa), high sensitivity (~100 fmol), high accuracy (~0.1%), and high resolution (5000 full-width at half-maximum intensity).

Identification of Individual Molecular Species of Sphingolipid by DE MALDI-TOF MS

Because any one class of sphingolipid consists of many individual molecular species by the combination of long-chain bases (d18:1 and d20:1 sphingosine, d18:0 and d20:0 dihydrosphingosine, and t18:0 and t20:0 phytosphingosine, etc.) and a variety of fatty acids (unbranched, monounsaturated and α-hydroxy alkyl chain lengths from 14 to 28 carbon atoms), the isolation and identification of individual molecular species of sphingolipid have previously been impossible. DE MALDI-TOF MS is capable of giving rise to molecular weight-related ions corresponding to the molecular species, and the numerous molecular ions can be identified in combination of these results with other analyses of the long-chain bases in the form of the lysosphingolipids prepared by deacylation of the starting sphingolipid.

Preparation of Lysosphingolipids

Because our usual method[3–5] for the preparation of lysosphingolipids was not optimized for microanalysis, a new method has been invented using

[3] T. Taketomi and T. Yamakawa, *J. Biochem.* **54**, 444 (1963).
[4] T. Taketomi and N. Kawamura, *J. Biochem.* **68,** 475 (1970).
[5] T. Taketomi, N. Kawamura, A. Hara, and S. Murakami, *Biochim. Biophys. Acta* **424,** 106 (1970).

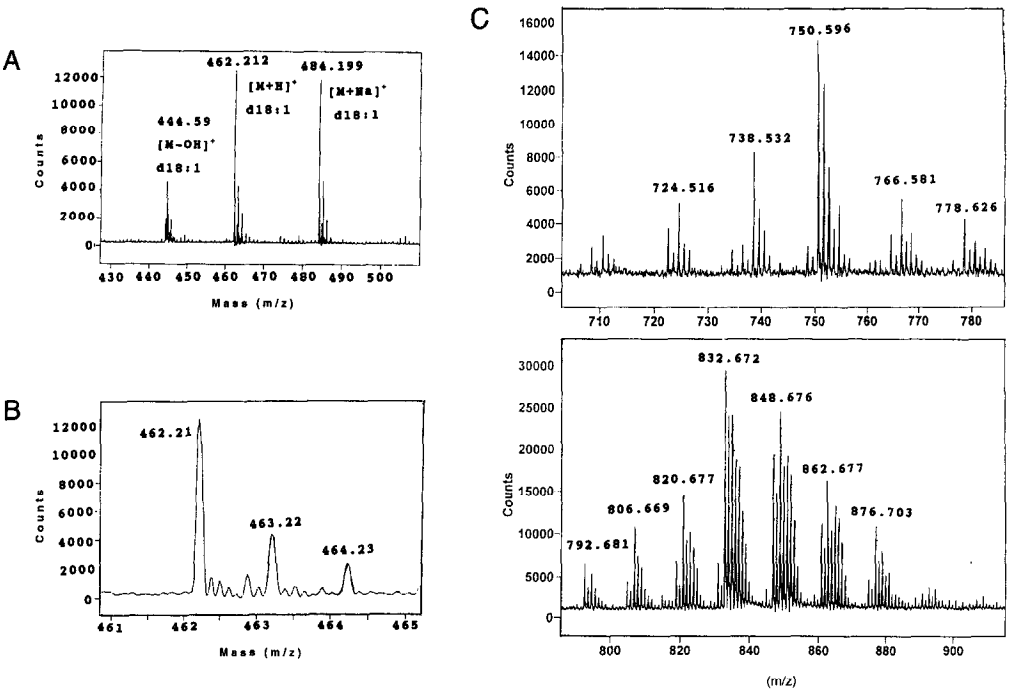


FIG. 1. (A) DE MALDI-TOF mass spectrum of psychosine deacylated from galactosylceramide (cerebroside of monkey brain) in the positive ion mode. (B) Enlarged high-resolution spectrum of isotopically resolved peaks of protonated psychosine (d18:1); $[M + H]^+$ at m/z 462.21 in (A) for the determination of psychosine (d18:0). (C) DE MALDI-TOF mass spectrum of intact cerebroside (monkey brain).

Mass (m/z)	Ions	LCB	FA	Mass (m/z)	Ions	LCB	FA
710	$[M + Na]^+$	d18:0	C15:0	820	$[M + Na]^+$	d18:1	C23:0
722	$[M + Na]^+$	d18:1	C16:0	822	$[M + Na]^+$	d18:0	C23:0
724	$[M + Na]^+$	d18:0	C16:0		$[M + Na]^+$	d18:1	C22:0h
736	$[M + Na]^+$	d18:1	C17:0	832	$[M + Na]^+$	d18:1	C24:1
738	$[M + Na]^+$	d18:0	C17:0	834	$[M + Na]^+$	d18:1	C24:0
750	$[M + Na]^+$	d18:1	C18:0	836	$[M + Na]^+$	d18:0	C24:0
754	$[M + H]^+$	d18:1	C20:1		$[M + Na]^+$	d18:1	C23:0h
764	$[M + Na]^+$	d18:1	C85:0h	846	$[M + Na]^+$	d18:1	C25:1
766	$[M + Na]^+$	d18:0	C18:0h	848	$[M + Na]^+$	d18:1	C25:0
768	$[M + H]^+$	d18:1	C20:0h		$[M + Na]^+$	d18:1	C24:1h
778	$[M + Na]^+$	d18:1	C20:0	850	$[M + Na]^+$	d18:1	C24:0h
780	$[M + H]^+$	d18:1	C22:0		$[M + Na]^+$	d18:0	C24:1h
782	$[M + H]^+$	d18:0	C22:0	860	$[M + Na]^+$	d18:1	C26:1
792	$[M + Na]^+$	d18:1	C20:1h	862	$[M + Na]^+$	d18:1	C26:0
794	$[M + Na]^+$	d18:1	C20:0h	864	$[M + Na]^+$	d18:0	C26:0
804	$[M + Na]^+$	d18:1	C22:1	874	$[M + Na]^+$	d18:1	C27:1
806	$[M + Na]^+$	d18:1	C22:0	876	$[M + Na]^+$	d18:1	C27:0
818	$[M + Na]^+$	d18:1	C23:1	878	$[M + Na]^+$	d18:0	C27:0

LCB, long-chain base; FA, fatty acid; h, hydroxy fatty acid.

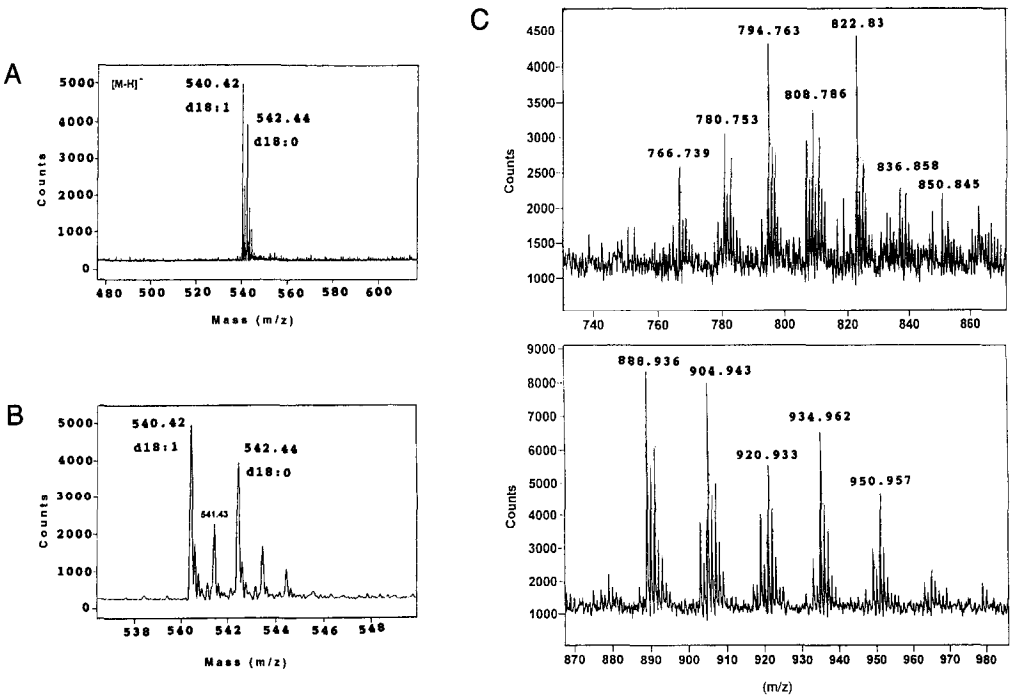


FIG. 2. (A) DE MALDI-TOF mass spectrum of lysosulfatide deacylated from sulfatide (monkey brain) in the negative ion mode. (B) Enlarged high-resolution spectrum of isotopically resolved peaks of deprotonated lysosulfatide (d18:1), $[M - H]^-$ at m/z 540.42 in (A) for the determination of lysosulfatide (d18:0). (C) DE MALDI-TOF mass spectrum of intact sulfatide (monkey brain).

Mass (m/z)	Ions	LCB	FA	Mass (m/z)	Ions	LCB	FA
765	$[M - H]^-$	d18:1	C15:0	889	$[M - H]^-$	d18:1	C24:1
767	$[M - H]^-$	d18:0	C15:0	891	$[M - H]^-$	d18:1	C24:0
779	$[M - H]^-$	d18:1	C16:0	903	$[M - H]^-$	d18:1	C25:1
781	$[M - H]^-$	d18:0	C16:0	905	$[M - H]^-$	d18:1	C25:0
783	$[M - H]^-$	d18:0	C15:0h		$[M - H]^-$	d18:1	C24:1h
793	$[M - H]^-$	d18:1	C17:0	907	$[M - H]^-$	d18:0	C25:0
795	$[M - H]^-$	d18:1	C16:0h		$[M - H]^-$	d18:1	C24:0h
797	$[M - H]^-$	d18:0	C16:0h	919	$[M - H]^-$	d18:1	C26:0
807	$[M - H]^-$	d18:1	C18:0		$[M - H]^-$	d18:1	C25:1h
809	$[M - H]^-$	d18:0	C18:0	921	$[M - H]^-$	d18:0	C26:0
811	$[M - H]^-$	d18:0	C17:0h		$[M - H]^-$	d18:1	C25:0h
823	$[M - H]^-$	d18:1	C18:0h	933	$[M - H]^-$	d18:1	C27:0
825	$[M - H]^-$	d18:0	C18:0h		$[M - H]^-$	d18:1	C26:1h
833	$[M - H]^-$	d18:1	C20:0	935	$[M - H]^-$	d18:0	C27:0
837	$[M - H]^-$	d18:0	C20:0		$[M - H]^-$	d18:1	C26:0h
839	$[M - H]^-$	d18:0	C19:0h	949	$[M - H]^-$	d18:0	C28:0
851	$[M - H]^-$	d18:1	C20:0h	951	$[M - H]^-$	d18:1	C27:0h
879	$[M - H]^-$	d18:0	C23:0	965	$[M - H]^-$	d18:0	C28:0h
	$[M - H]^-$	d18:1	C22:0h				

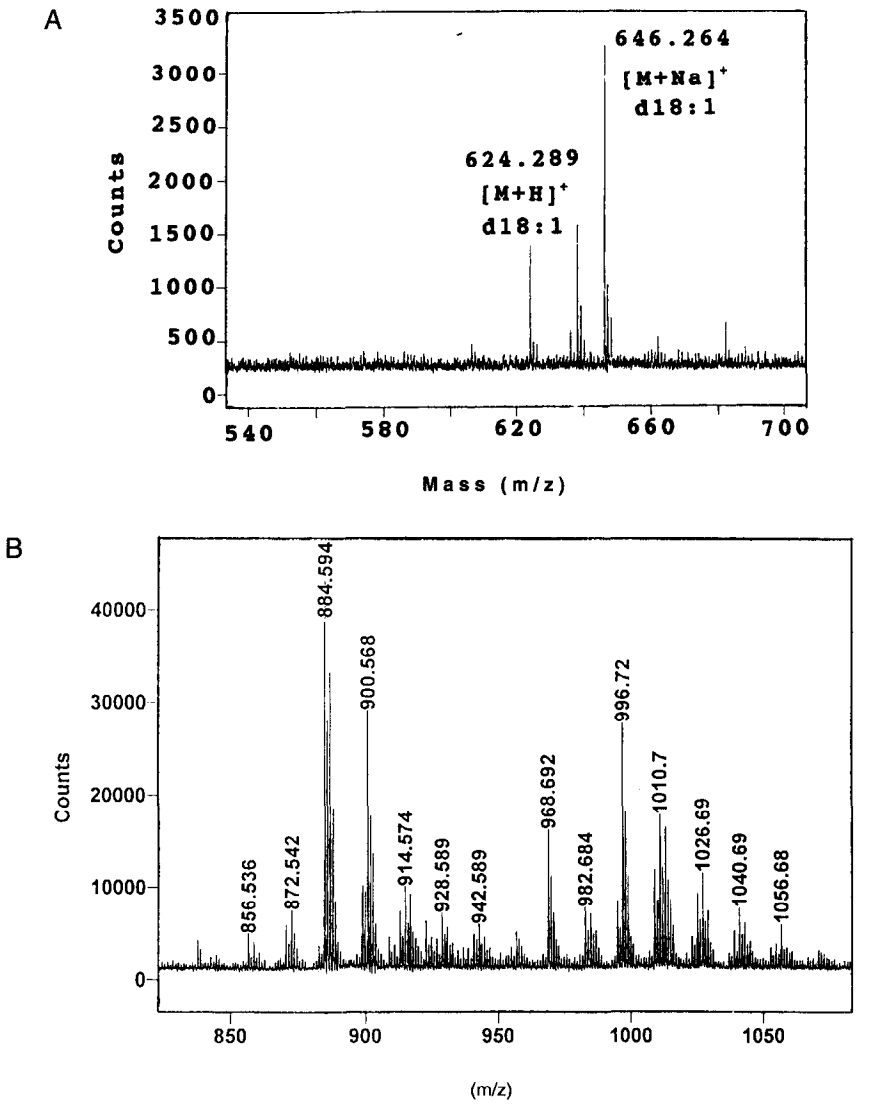


FIG. 3. (A) DE MALDI-TOF mass spectrum of lysolactosylceramide deacylated from lactosylceramide (porcine erythrocytes) in the positive ion mode. Protonated and sodium-adducted lysolactosylceramide (d18:1), $[M + H]^+$ at m/z 624.29 and $[M + Na]^+$ at m/z 646.26. (B) DE MALDI-TOF mass spectrum of intact lactosylceramide (porcine erythrocytes) in the positive ion mode.

microwave-mediated saponification of glycosphingolipids except sphingomyelin.[6–8] This is carried out as follows: about 1 mg of glycosphingolipids is dissolved in 0.5 ml of 0.1 *M* NaOH in methanol in a thick-walled Pyrex glass centrifuge tube (10 ml) with a Teflon-lined screw cap. The atmosphere is replaced by argon (or nitrogen) and the tubes are irradiated in a microwave oven (500 W, Toshiba model ER-V-11) for 2 min. For some sphingolipids, the yield is improved by conducting the microwave irradiation while the sample is under an atmosphere of argon (personal communication with Dr. Alfred H. Merrill). The volume of the alkaline methanol solution must not exceed 0.5 ml and other safety precautions should be taken because the tube may explode. A sealed glass tube must not be used owing to the possibility of explosion of the tube.

After saponification, the slightly turbid solution is cooled to room tem-

[6] T. Taketomi, A. Hara, K. Uemura, and E. Sugiyama, *J. Biochem.* **120,** 573 (1996).
[7] T. Taketomi, A. Hara, K. Uemura, H. Kurahashi, and E. Sugiyama, *Biochem. Biophys. Res. Commun.* **224,** 462 (1996).
[8] T. Taketomi, A. Hara, K. Uemura, H. Kurahashi, and E. Sugiyama, *J. Biochem.* **121,** 264 (1997).

FIG. 3. (*continued*)

Mass (*m/z*)	Ions	LCB	FA	Mass (*m/z*)	Ions	LCB	FA
856	$[M + Na]^+$	d18:1	C14:0	984	$[M + Na]^+$	d18:0	C23:0
872	$[M + Na]^+$	d18:0	C15:0		$[M + Na]^+$	d18:1	C22:0h
884	$[M + Na]^+$	d18:1	C16:0	986	$[M + Na]^+$	d18:0	C22:0h
886	$[M + Na]^+$	d18:0	C16:0	994	$[M + Na]^+$	d18:1	C24:1
898	$[M + Na]^+$	d18:1	C17:0	996	$[M + Na]^+$	d18:1	C24:0
900	$[M + Na]^+$	d18:1	C16:0h	1008	$[M + Na]^+$	d18:1	C25:1
	$[M + Na]^+$	d18:0	C17:0	1010	$[M + Na]^+$	d18:1	C25:0
912	$[M + Na]^+$	d18:1	C18:0	1012	$[M + Na]^+$	d18:0	C25:0
914	$[M + Na]^+$	d18:0	C18:0		$[M + Na]^+$	d18:1	C24:0h
	$[M + Na]^+$	d18:1	C17:0h	1022	$[M + Na]^+$	d18:1	C26:1
916	$[M + Na]^+$	d18:0	C17:0h	1024	$[M + Na]^+$	d18:1	C26:0
928	$[M + Na]^+$	d18:1	C18:0h	1026	$[M + Na]^+$	d18:0	C26:0
	$[M + Na]^+$	d18:0	C19:0		$[M + Na]^+$	d18:1	C25:0h
930	$[M + Na]^+$	d18:0	C18:0h	1028	$[M + Na]^+$	d18:0	C25:0h
940	$[M + Na]^+$	d18:1	C20:1	1038	$[M + Na]^+$	d18:1	C27:0
942	$[M + Na]^+$	d18:1	C20:0	1040	$[M + Na]^+$	d18:0	C27:0
	$[M + Na]^+$	d18:0	C20:1		$[M + Na]^+$	d18:1	C26:0h
944	$[M + Na]^+$	d18:0	C20:0	1042	$[M + Na]^+$	d18:0	C26:0h
968	$[M + Na]^+$	d18:1	C22:0	1052	$[M + Na]^+$	d18:1	C28:0
	$[M + Na]^+$	d18:0	C22:1	1054	$[M + Na]^+$	d18:0	C28:0
982	$[M + Na]^+$	d18:1	C23:0	1056	$[M + Na]^+$	d18:0	C27:0h

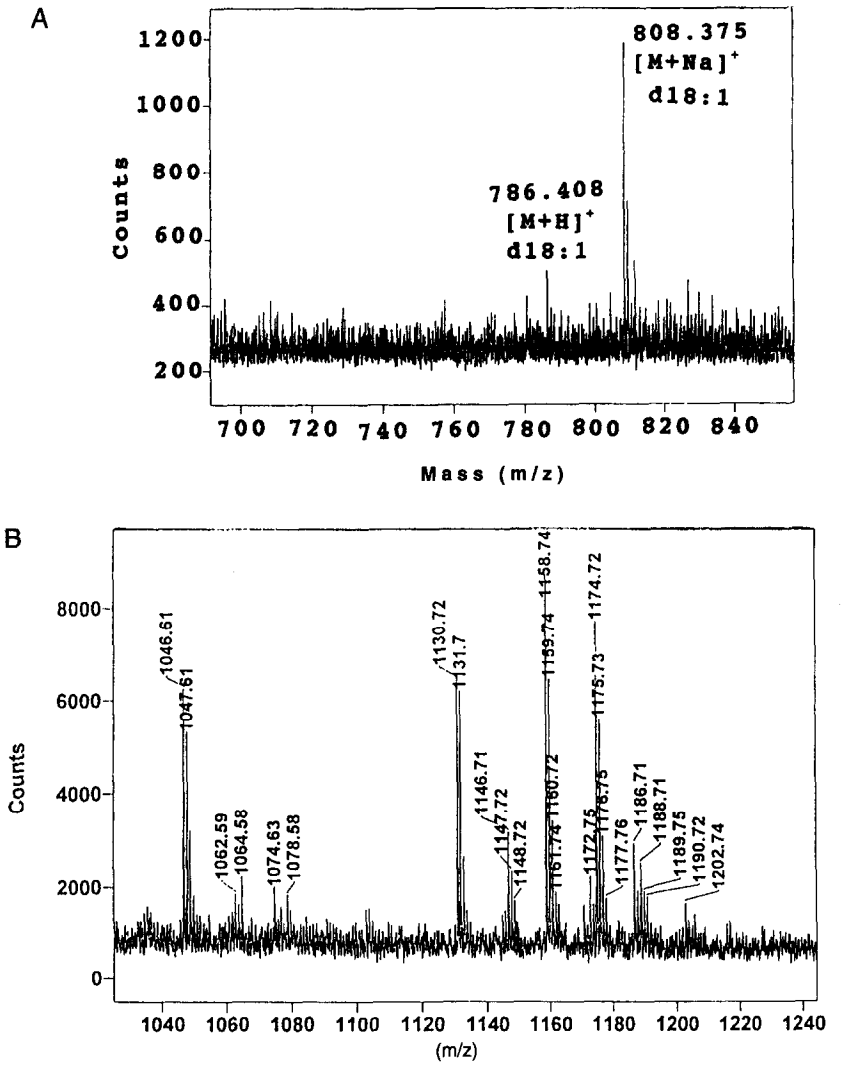


FIG. 4. (A) DE MALDI-TOF mass spectrum of lysoglobotriaosylceramide deacylated from globotriaosylceramide (porcine erythrocytes) in the positive ion mode. Protonated and sodium-adducted lysoglobotriaosylceramide (d18:1), $[M + H]^+$ at m/z 786.41 and $[M + Na]^+$ at m/z 808.37. (B) DE MALDI-TOF mass spectrum of globotriaosylceramide (porcine erythrocytes) in the positive ion mode.

perature, and acidified with one drop of 3 *N* HCl together with one drop of distilled water. The reaction mixture is vigorously shaken with 1 ml of hexane using a vortex mixer and is allowed to separate into an upper phase and a lower phase. The upper phase contains free fatty acids and is removed carefully with a micropipette. After the lower phase is extracted with hexane, it is applied to a Sep-Pak C_{18} cartridge (Waters Associates, Milford, MA) to remove salt. The cartridge is washed with about 5 ml of methanol–water (3:7, v/v), 5 ml of water, and then the lysoglycosphingolipids (and occasionally unreacted glycosphingolipids) are eluted with about 5 ml of ethanol–water (8:2, v/v) and 5 ml of chloroform–methanol–water (60:30:4.5, v/v/v). After the solvent has been evaporated under N_2, the lyso compound is subjected to DE MALDI-TOF MS analysis for the confirmation of lysoglycosphingolipid as well as the determination of long-chain base composition.

Analysis of Lysosphingolipids and Sphingolipids by DE MALDI-TOF MS

The mass spectrometric analysis is performed as follows[6–9]: One microliter of chloroform–methanol (1:1, v/v) solution containing about 1–10 pmol of lysoglycosphingolipid or 50–200 pmol of sphingolipid and 1 μl of the matrix solution [10 mg of α-cyano-4-hydroxycinnamic acid (α-CHCA) in 1 ml of a 1:1 mixture of acetonitrile–water containing 0.1% of trifluoroacetic acid, or 10 mg of 2,5-dihydroxybenzoic acid (2,5-DHB) in 1 ml of 8:2 mixture of water–ethanol] are placed in a 1.5-ml Eppendorf tube, shaken vigorously on a vortex mixer, and then spun down in a microcentrifuge (Chibitan, Japan Millipore, Tokyo, Japan).

[9] E. Sugiyama, A. Hara, K. Uemura, and T. Taketomi, *Glycobiology* **7**, 719 (1997).

FIG. 4. (*continued*)

Mass (*m/z*)	Ions	LCB	FA	Mass (*m/z*)	Ions	LCB	FA
1046	$[M + Na]^+$	d18:1	C16:0	1158	$[M + Na]^+$	d18:1	C24:0
1062	$[M + Na]^+$	d18:1	C16:0h	1174	$[M + Na]^+$	d18:1	C24:0h
1064	$[M + Na]^+$	d18:0	C16:0h		$[M + Na]^+$	d18:0	C24:1h
1074	$[M + Na]^+$	d18:1	C18:0	1186	$[M + Na]^+$	d18:1	C25:1h
1078	$[M + H]^-$	d18:1	C20:1	1188	$[M + Na]^+$	d18:1	C25:0
1130	$[M + Na]^+$	d18:1	C22:0		$[M + Na]^+$	d18:0	C25:1h
1146	$[M + H]^+$	d18:1	C22:0h	1202	$[M + Na]^+$	d18:0	C26:1h
1158	$[M + Na]^+$	d18:0	C24:1		$[M + Na]^+$	d18:1	C26:0h

One microliter of the supernatant is loaded onto the sample plate, then allowed to dry and crystallize for about 20 min at room temperature. The sample plate is loaded into the load position of Voyager Elite XL (6.6-m flight length in the reflector mode) Biospectrometry Workstation (PerSeptive Biosystem, Framingham, MA). A nitrogen laser (337 nm) is used for the desorption ionization. In the positive ion mode with 2,5-DHB or α-CHCA used as the matrix, potassium ($[M]^+$: 38.96) and angiotensin I ($[M + Na]^+$: 1,297.50) are used to calibrate the instrument. Neutral glycosphingolipids and sphingomyelin are usually measured in the positive ion mode. In the negative ion mode with 2,5-DHB or α-CHCA, lysosulfatide (d18:1, $[M - H]^-$: 540.28) and GM1 (d18:1, C_{18}, $[M - H]^-$: 1544.87) are used to calibrate the instrument. Acidic glycosphingolipids are measured in the negative ion made. An adequate N_2 laser step (1400–2800) was freely selected for a good analytical result. The resolution of the ion peak (full-width at half-maximum intensity) is measured by the resolution calculator in the GRAM/386 software. Five-point Savitsky–Golay smoothing is usually applied to the spectra.

Gala-Series Glycosphingolipids

A lyso compound is prepared from galactosylceramide (cerebroside) isolated from monkey brain and it, as well as the intact cerebroside, is separately subjected to DE MALDI-TOF MS analysis.[7] The mass spectrum in Fig. 1A shows molecular weight-related ions of $[M - OH]^+$ at m/z 444.59, $[M + H]^+$ at m/z 462.21, and $[M + Na]^+$ at m/z 484.20, confirming psychosine (lysogalactosylceramide) containing d18:1 mainly. Also, the presence of psychosine containing d18:0 was detected by the following procedure.

The counts of the isotopically resolved ion peaks at m/z 462.21, 463.22, and 464.23 in the enlarged high-resolution spectrum in Fig. 1B accounted for 65.9, 22.5, and 11.6%. The probabilities of the corresponding peaks indicating no ^{13}C atoms (monoisotopic molecule), one ^{13}C atom, and two ^{13}C atoms of psychosine (d18:1) were theoretically calculated by the natural abundance of stable isotope as 76.7, 20.6, and 2.7%.

Because the difference between the found and theoretical values indicated the presence of psychosine (d18:0), the long-chain base composition of monkey brain cerebroside was estimated to be d18:1 (91.1%) and d18:0 (8.9%). Thus, the molecular weight-related ion peaks corresponding to individual molecular species of monkey brain cerebroside in the mass spectrum were identified as described in the legend to Fig. 1C. Although this analysis of the mass spectrum provides a quantitative analysis of individual

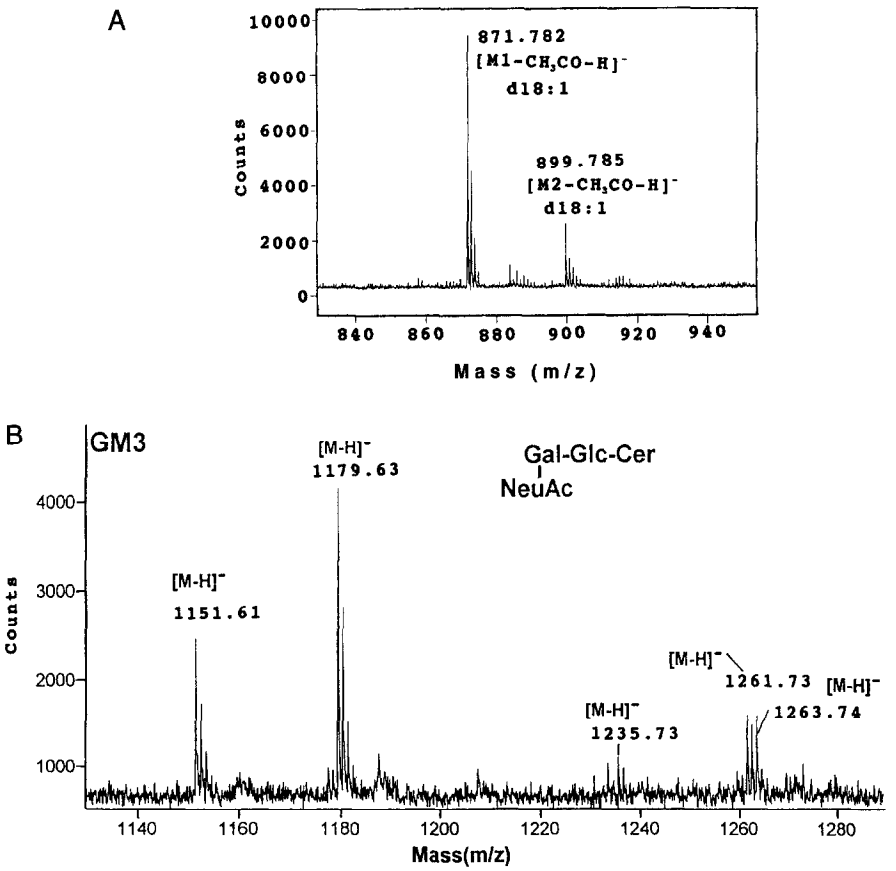
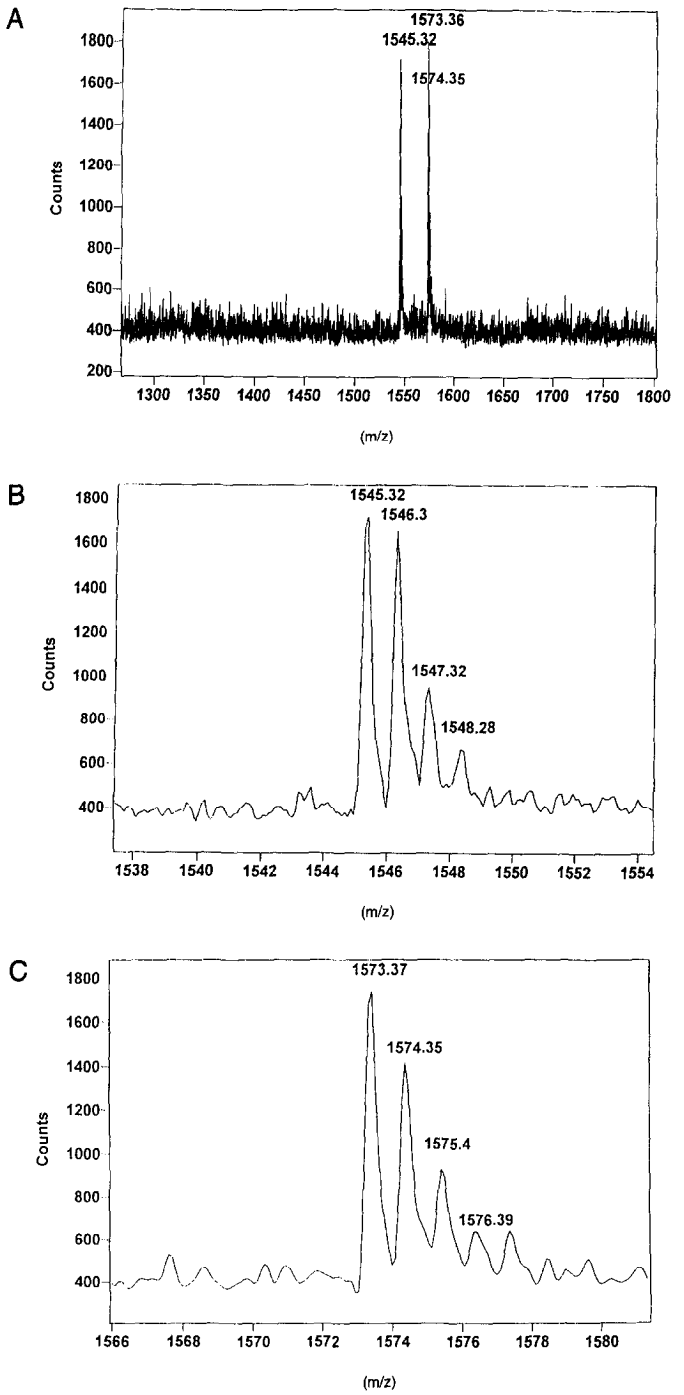


FIG. 5. (A) DE MALDI-TOF mass spectrum of the lyso compound deacylated from GM3 (human brain tumor) in the negative ion mode. Deprotonated deacetylated lysoGM3 (d18:1) and (d20:1), $[M_1\text{-}CH_3CO\text{-}H]^-$ at m/z 871.78 and $[M_2\text{-}CH_3CO\text{-}H]^-$ at m/z 899.79. (B) DE MALDI-TOF mass spectrum of intact GM3 (human brain tumor) in the negative ion mode.

Mass (m/z)	Ions	LCB	FA	Mass (m/z)	Ions	LCB	FA
1151	$[M - H]^-$	d18:1	C16:0	1261	$[M - H]^-$	d18:1	C24:1
1179	$[M - H]^-$	d18:1	C18:0		$[M - H]^-$	d20:1	C22:1
1235	$[M - H]^-$	d18:1	C20:0	1263	$[M - H]^-$	d18:1	C24:0
	$[M - H]^-$	d20:1	C18:0		$[M - H]^-$	d20:1	C22:0


A
1573.36
1545.32
1574.35
Counts
1800
1600
1400
1200
1000
800
600
400
200
1300 1350 1400 1450 1500 1550 1600 1650 1700 1750 1800
(m/z)
B
1545.32
1546.3
1547.32
1548.28
Counts
1800
1600
1400
1200
1000
800
600
400
1538 1540 1542 1544 1546 1548 1550 1552 1554
(m/z)
C
1573.37
1574.35
1575.4
1576.39
Counts
1800
1600
1400
1200
1000
800
600
400
1566 1568 1570 1572 1574 1576 1578 1580
(m/z)

molecular species, the ability of this procedure to give exact quantities awaits.

Figure 2A shows the mass spectrum of the lyso compound obtained from another class of sphingolipid, sulfatide prepared from monkey brain provided a molecular weight-related ion of $[M - H]^-$ at m/z 540.42, confirming lysosulfatide (d18:1). The presence of lysosulfatide (d18:0) was also confirmed by the difference between the found and theoretical values of the isotopically resolved peaks in the enlarged high-resolution spectrum in Fig. 2B, thus, the long-chain base composition of monkey brain sulfatide was estimated to be d18:1 (70.6%) and d18:0 (29.4%). The molecular weight-related ions corresponding to individual molecular species of monkey brain sulfatide were identified as shown in the legend to Fig. 2C.

Globo-Series Glycosphingolipids

Globo-series lysoglycosphingolipids[7] are analyzed by this same procedure. Lysolactosylceramide was prepared from porcine erythrocytes and gave the molecular weight-related ions $[M + H]^+$ at m/z 624.29 and $[M + Na]^+$ at m/z 646.26 in the mass spectrum in Fig. 3A, and the ratio of d18:1 to d18:0 in the latter ion peak was estimated to be 95.8 and 4.2%. Thus, the molecular weight-related ion peaks corresponding to individual molecular species of lactosylceramide were decided as shown in the legend to Fig. 3B. Lysoglobotriaosylceramide from the same source gave the molecular weight-related ions $[M + H]^+$ at m/z 786.41 and $[M + Na]^+$ at m/z 808.38 in the mass spectrum in Fig. 4A, and the ratio of d18:1 to d18:0 in the latter peak was estimated to be 97.8 and 2.2%. Thus, the molecular weight-related ion peaks corresponding to individual molecular species of globotriaosylceramide were determined as shown in the legend to Fig. 4B. The same approach can be applied to lysoglobotetraosylceramide and lysoglobopentaosylceramide.[6]

Ganglio-Series Glycosphingolipids: Gangliosides and Asialogangliosides

Gangliosides[5,9] are highly complex oligoglycosylceramides, which contain one or more sialic acid groups, in addition to glucose, galactose, and

FIG. 6. (A) DE MALDI-TOF mass spectrum of intact GM1 (adult human brain) in the negative ion mode. Molecular weight-related ions: $[M_1 - H]^-$ at m/z 1545.32 and $[M_2 - H]^-$ at m/z 1573.36 corresponding to GM1 (d18:1C18:0) and GM1 (d20:1C18:0). (B) Enlarged high-resolution spectrum of the isotopically resolved peaks of deprotonated GM1 (d18:1C18:0) for the determination of GM1 (d18:0C18:0). (C) Enlarged high-resolution spectrum of the isotopically resolved peaks of deprotonated GM1 (d20:1C18:0) for the determination of GM1 (d20:0C18:0).

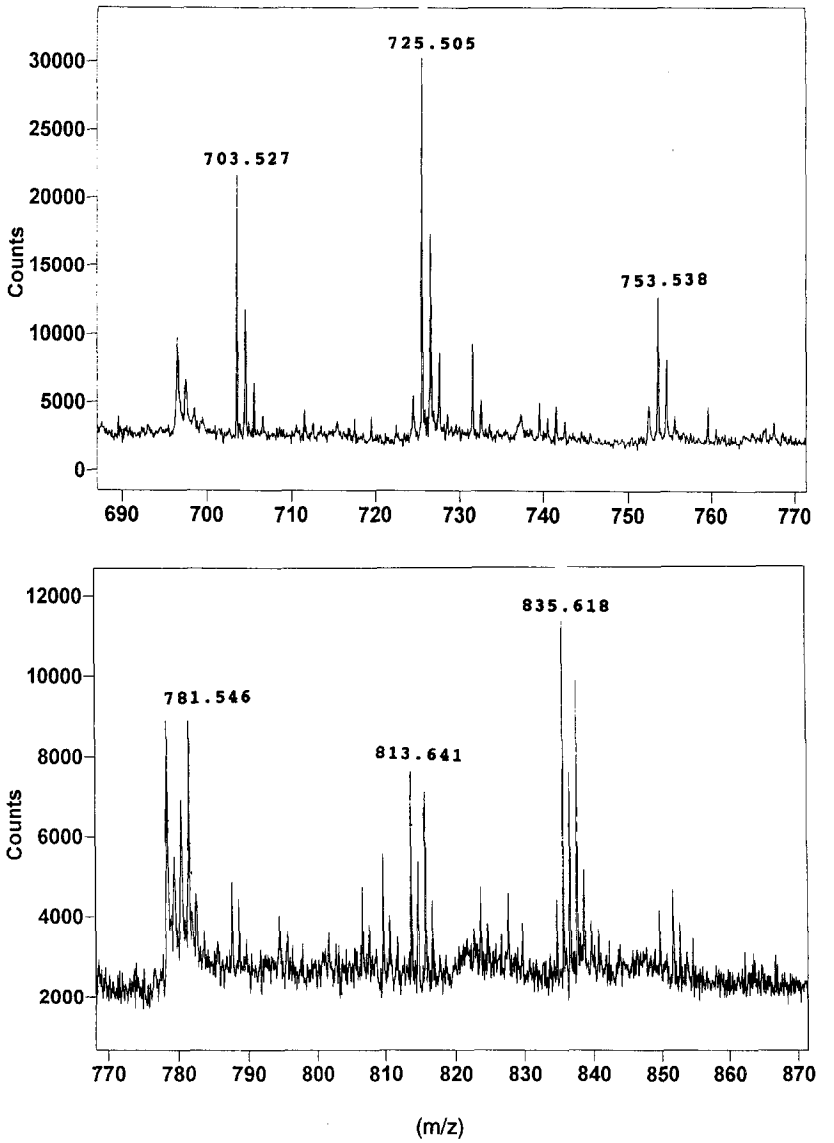


FIG. 7. DE MALDI-TOF mass spectrum of intact sphingomyelin (caprine erythrocytes) in the positive ion mode.

N-acetylgalactosamine. However, most gangliosides contain a major long-chain base of d18:1 and d20:1 and major stearic acid and thus consist of relatively simple individual molecular species.

Regarding GM3 isolated from human brain tumor tissue,[9] the lyso compound prepared from GM3 gave two molecular weight-related ion peaks corresponding to lysoGM3 (d18:1), $[M_1\text{-}CH_3CO\text{-}H]^-$ at m/z 871.78 and lysoGM3 (d20:1), $[M_2\text{-}CH_3CO\text{-}H]^-$ at m/z 899.79 in the mass spectrum in negative ion mode in Fig. 5A. Also, the presence of lysoGM3 (d18:0) and (d20:0) was detected by the difference between the found and theoretical values of three isotopically resolved peaks of each lysoGM3 (d18:1) and (d20:1) in both the enlarged high-resolution spectra (data not shown) and thus the ratios of d18:1 to d18:0 and d20:1 to d20:0 were estimated to be 97.6 and 2.4%, and 99.1 and 0.9%. Together with the percentage of counts of two molecular weight-related ions of lysoGM3 (d18:1) and (d20:1), the long-chain base composition of GM3 was estimated to be d18:1 (77.4%), d18:0 (1.9%), d20:1 (20.5%), and d20:0 (0.2%). Thus, the molecular weight-related ion speaks corresponding to individual molecular species of GM3 of human brain tumor tissue were decided as shown in the legend to Fig. 5B. Analysis of GM2 can be made by this same method.

Finally, regarding GM1 from the brain tissue,[10] the preparation of lyso compound is not always necessary for the determination of the long-chain

[10] T. Taketomi, A. Hara, K. Uemura, and E. Sugiyama, *Acta Biochim. Polon.* **45,** 987 (1998).

FIG. 7. (*continued*)

Mass (m/z)	Ions	LCB	FA	Mass (m/z)	Ions	LCB	FA
696	?	?	?	781	$[M + Na]^+$	d18:1	C20:0
697	$[M + Na]^+$	d18:1	C14:0	806	?	?	?
703	$[M + H]^+$	d18:1	C16:0	809	$[M + Na]^+$	d18:1	C22:0
711	$[M + Na]^+$	d18:1	C15:0	813	$[M + H]^+$	d18:1	C24:1
725	$[M + Na]^+$	d18:1	C16:0	815	$[M + H]^+$	d18:1	C24:0
*	$[M + Na]^+$	d18:0	C16:1	823	$[M + Na]^+$	d18:1	C23:0
731	$[M + H]^+$	d18:1	C18:0	827	$[M + H]^+$	d18:1	C25:1
739	$[M + K]^+$	d18:1	C16:0	835	$[M + Na]^+$	d18:1	C24:1
741	$[M + K]^+$	d18:0	C16:0	837	$[M + Na]^+$	d18:1	C24:0
753	$[M + Na]^+$	d18:1	C18:0	**	$[M + Na]^+$	d18:0	C24:1
759	$[M + H]^+$	d18:1	C20:0	849	$[M + Na]^+$	d18:1	C25:1
778	?	?	?	851	$[M + K]^+$	d18:1	C24:1

* and ** suggested by the isotopically resolved peaks of sphingomyelin $[M + Na]^+$ (d18:1C16:0) and (d18:1C24:0), respectively.

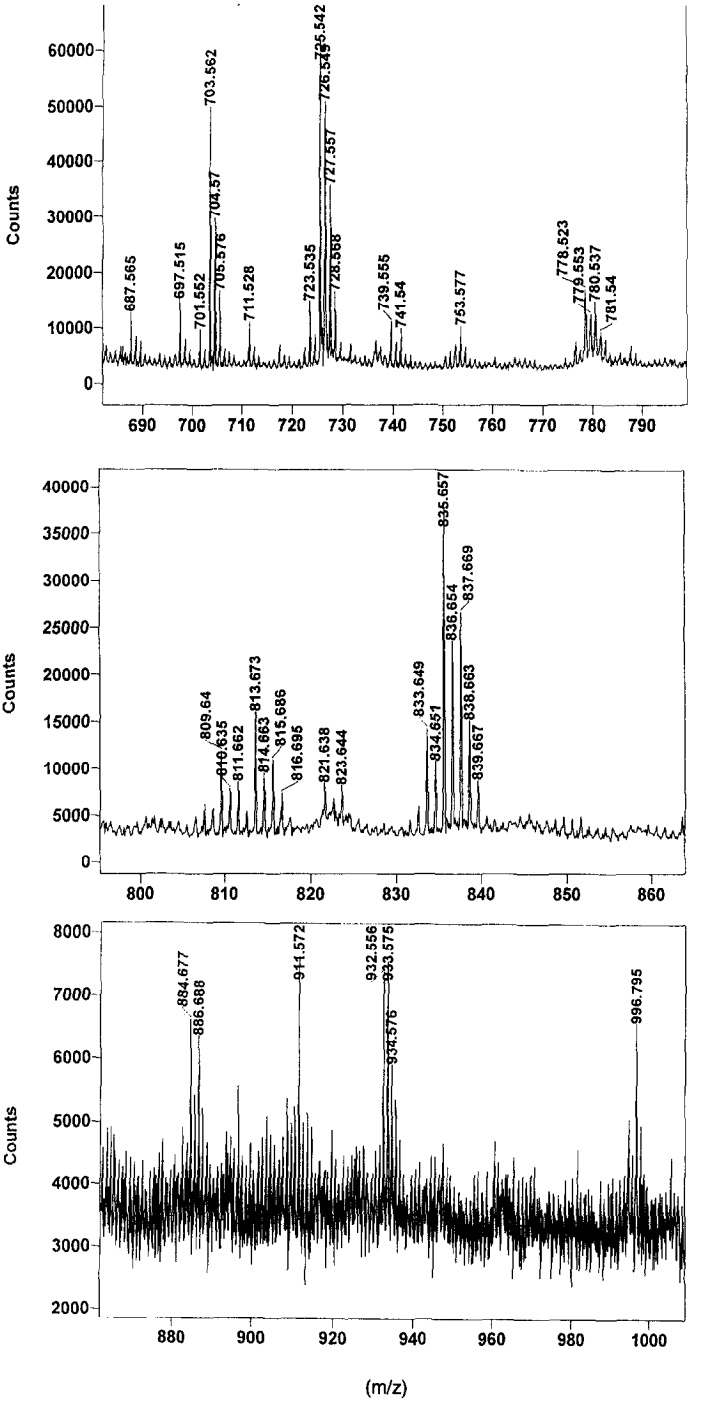


FIG. 8. DE MALDI-TOF mass spectrum of multiple sphingolipids of the alkali-stable lipid fraction from human platelets in the positive ion mode.

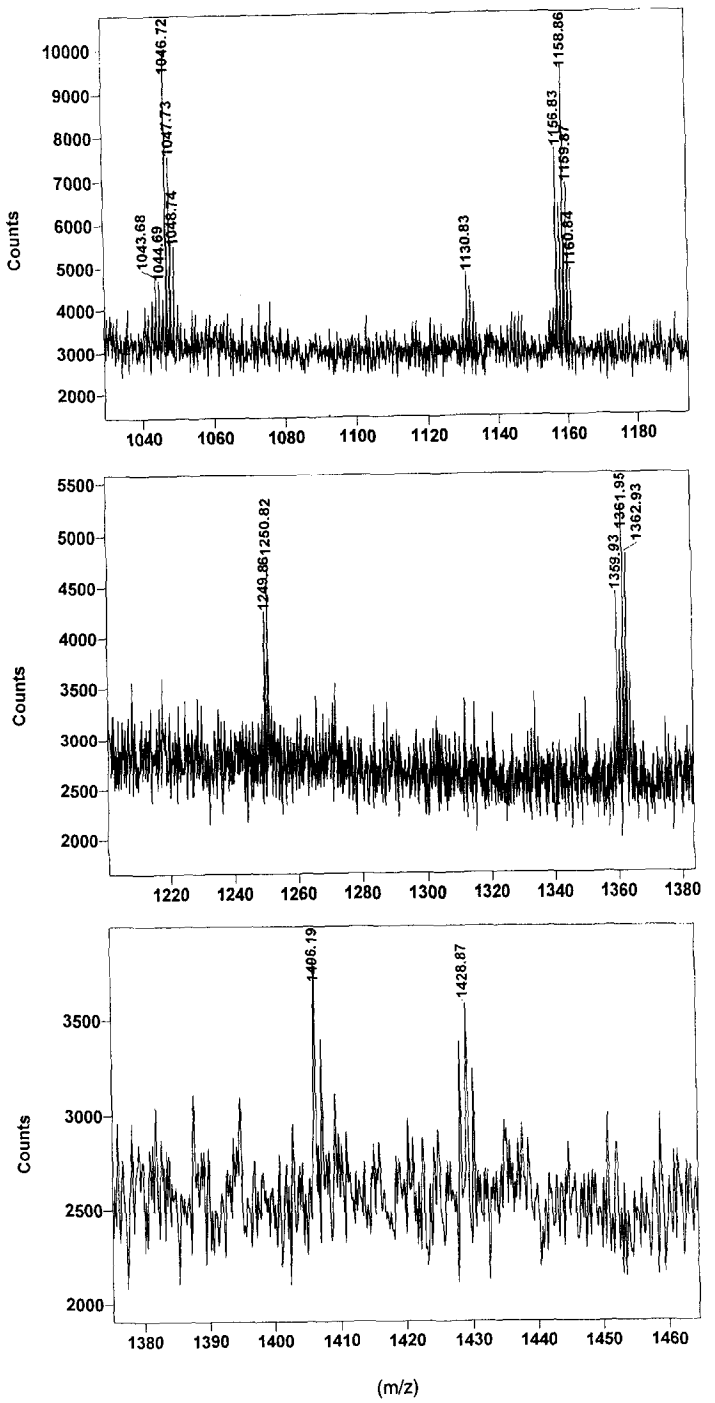


FIG. 8. (*continued*)

base composition, because there is only one fatty acid, stearic acid. Intact GM1 isolated from the brain tissue of an adult patient with sudanophilic leukodystrophy was directly analyzed by DE MALDI-TOF MS. As shown in Fig. 6A, it was exactly identified by two molecular weight-related ions: $[M_1 - H]^-$ at m/z 1545.42 corresponding to GM1 (d18:1C18:0) and $[M - H]^-$ at m/z 1573.46 corresponding to GM1 (d20:1C18:0). The GM1 (d18:0C18:0) and GM1 (d20:0C18:0) were also detected by the difference between the found and theoretical values of three isotopically resolved peaks of each GM1 (d18:1C18:0) and GM1 (d20:1C18:0) in both the enlarged high-resolution spectra in Figs. 6B and 6C in the same way as already described above, and the ratios of GM1 (d18:1C18) to (d18:0C18:0) and GM1 (d20:1C18:0) to (d20:0C18:0) were estimated to be 95.1 and 4.9%, and 93.4 and 6.6%. Together with the ratios of counts of GM1 (d18:1C18:0) to (d20:1C18:0), the long-chain base composition of GM1 was calculated to be d18:1 (44.2%), d18:0 (2.3%), d20:1 (49.9%), and d20:0 (3.6%). The

FIG. 8. (*continued*)

Mass (m/z)	Ions	LCB	FA	Mass (m/z)	Ions	LCB	FA
687	$SM[M + H]^+$	d18:1	C15:1	836	$SM[M + Na]^+$	d18:1	C24:1
697	$CMH[M + H]^+$	d18:1	C16:1	838	$SM[M + Na]^+$	d18:1	C24:0
703	$SM[M + H]^+$	d18:1	C16:0	884	$CDH[M + Na]^+$	d18:1	C16:0
	$SM[M + H]^+$	d18:0	C16:1	886	$CDH[M + Na]^+$	d18:0	C16:0
711	$SM[M + Na]^+$	d18:0	C15:0	911	$CDH[M + Na]^+$	d18:1	C18:0
723	$SM[M + Na]^+$	d18:1	C16:1	932	$CDH[M + H]^+$	d18:1	C21:0
725	$SM[M + Na]^+$	d18:1	C16:0	996	$CDH[M + Na]^+$	d18:1	C24:0
	$SM[M + Na]^+$	d18:0	C16:1	1046	$CTH[M + Na]^+$	d18:1	C16:0
727	$SM[M + Na]^+$	d18:0	C16:0	1131	$CTH[M + Na]^+$	d18:1	C22:0
739	$SM[M + K]^+$	d18:1	C16:1	1157	$CTH[M + Na]^+$	d18:1	C24:1
741	$SM[M + K]^+$	d18:1	C16:0	1159	$CTH[M + Na]^+$	d18:1	C24:0
753	$SM[M + Na]^+$	d18:1	C18:0	1250	$Gb[M + Na]^+$	d18:1	C16:0
778	$CMH[M + Na]^+$	d18:1	C20:0	1360	$Gb[M + Na]^+$	d18:1	C24:1
780	$CMH[M + Na]^+$	d18:0	C20:0	1362	$Gb[M + Na]^+$	d18:1	C24:0
809	$SM[M + Na]^+$	d18:1	C22:0	1406	$GM2[M + Na]^+$	d18:1	C18:0
811	$SM[M + Na]^+$	d18:0	C22:0	1428	$GM2[M + 2Na - H]^+$	d18:1	C18:0
813	$SM[M + H]^+$	d18:1	C24:1				
815	$SM[M + H]^+$	d18:1	C24:0				
821	$SM[M + Na]^+$	d18:1	C23:1				
823	$SM[M + Na]^+$	d18:1	C23:0				
834	$CMH[M + Na]^+$	d18:1	C24:0				

SM, sphingomyelin; CMH, glucosylceramide; CDH, lactosylceramide; CTH, globotriaosylceramide; Gb, globotetraosylceramide; GM2, ganglioside GM2.

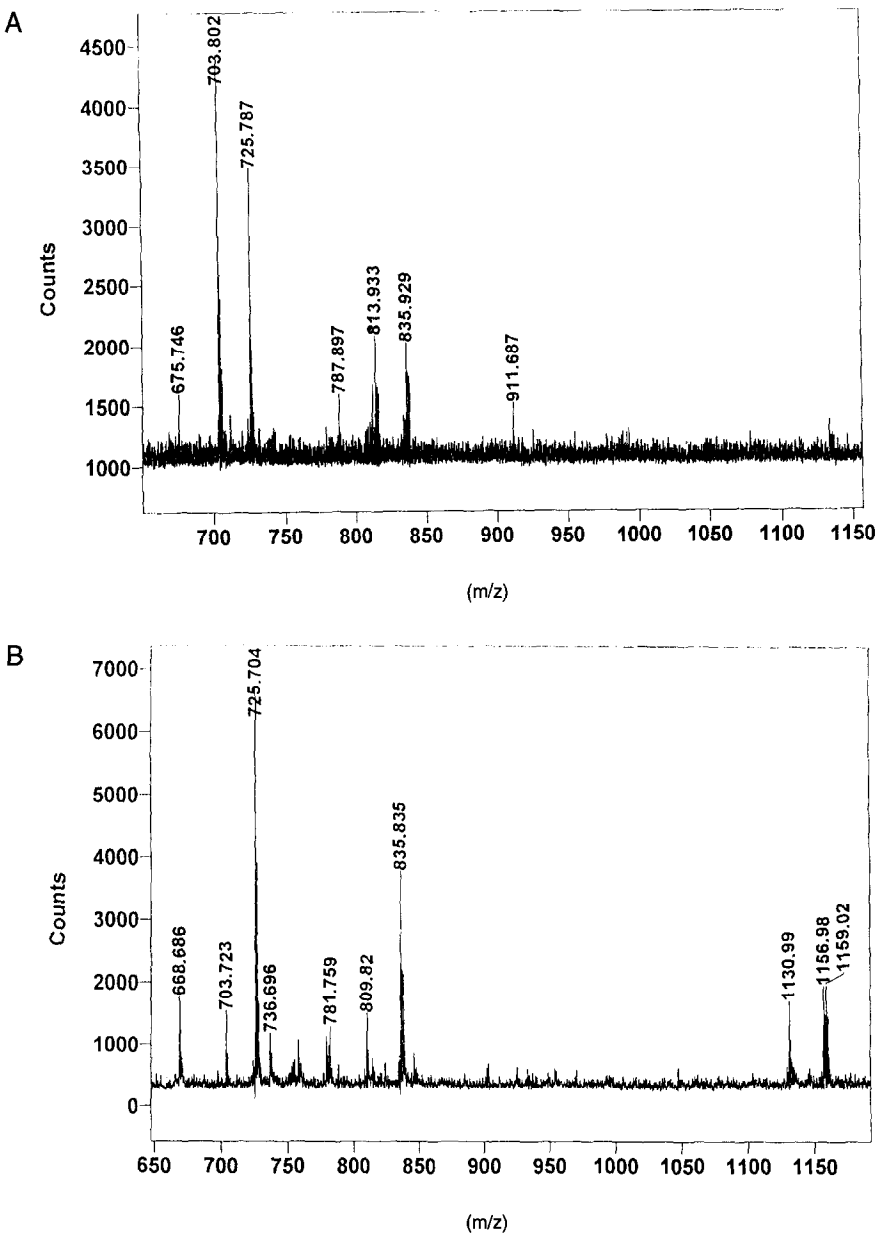


FIG. 9. (A) DE MALDI-TOF mass spectrum of multiple sphingolipids of the alkali-stable lipid fraction from the necrotic tissue of head of femur of a patient as a control in the positive ion mode. (B) DE MALDI-TOF mass spectrum of multiple sphingolipids of the alkali-stable lipid fraction from the necrotic tissue of head of femur of a patient with Fabry disease.

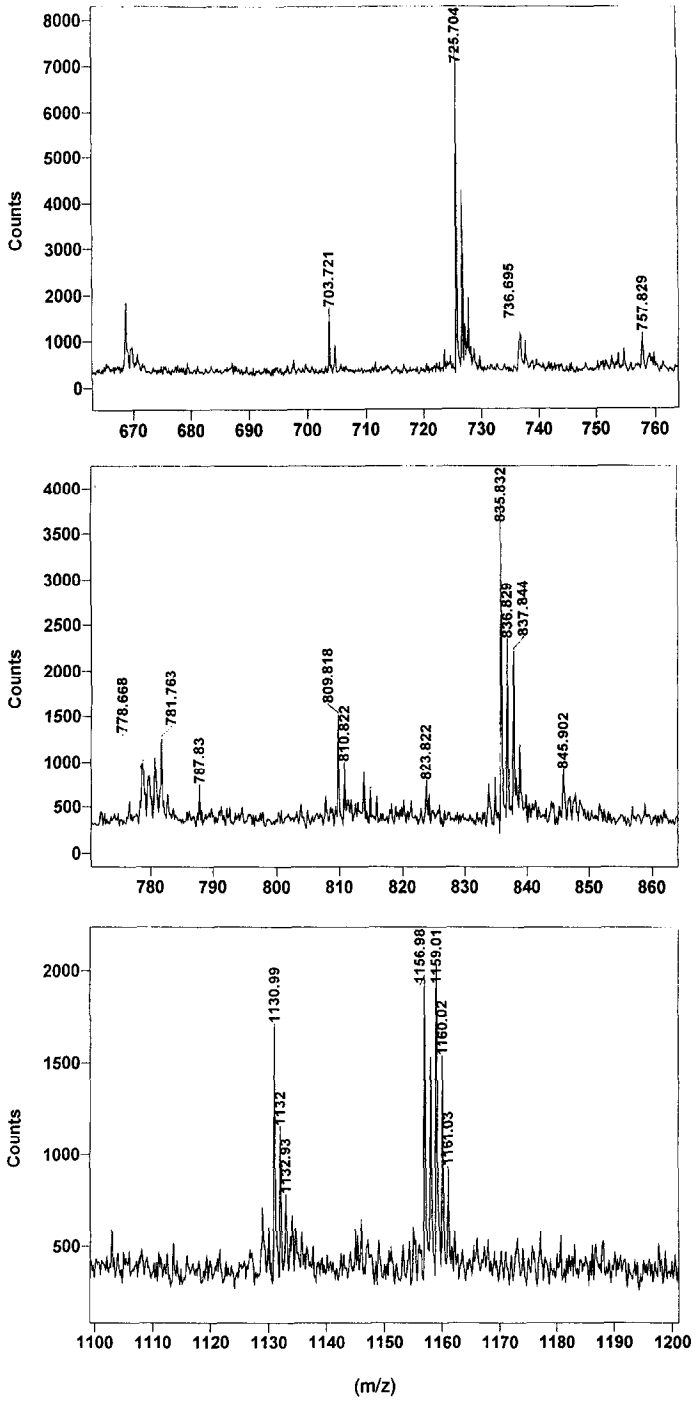


FIG. 10. Enlarged DE MALDI-TOF mass spectrum of Fig. 9B.

long-chain base compositions of other gangliosides containing major stearic acid can be determined in the same way by DE MALDI-TOF MS (data not shown).

Sphingomyelin

Because sphingomyelin in animal tissues has in general long-chain bases of d18:1 and d18:0 and unbranched and non-hydroxy fatty acids, its individual molecular species are relatively simple. When the intact sphingomyelin isolated from caprine erythrocytes was subjected to DE MALDI-TOF MS by the same procedure as described in the section on glycosphingolipids, the mass spectrum was found to indicate the molecular weight-related ions corresponding to the individual molecular species as shown in Fig. 7.

Extraction and DE MALDI-TOF MS Analysis of Multiple Sphingolipids from a Single Sample

DE MALDI-TOF MS analysis of sphingolipids is also possible with the alkali-stable lipid fraction obtained from a biologic sample. We have conducted the analysis with human platelets, human fibroblasts, sciatic nerve, and brain tissues of UDP-galactose ceramide galactosyltransferase (CGT) null mutant mouse ($ctg^{-/-}$) and wild-type mouse, and necrotic tissues from the head of a femur of a patient with Fabry disease and a control subject. A few examples are described in this section.

FIG. 10. (*continued*)

Mass (m/z)	Ions	LCB	FA	Mass (m/z)	Ions	LCB	FA
703	SM[M + H]$^+$	d18:1	C16:0	836	SM[M + Na]$^+$	d18:1	C24:1
725	SM[M + Na]$^+$	d18:1	C16:0	838	SM[M + Na]$^+$	d18:1	C24:0
737				846	SM[M + Na]$^+$	d18:1	C26:0
758	SM[M + Na]$^+$	d18:1	C20:1	1131	CTH[M + Na]$^+$	d18:1	C22:0
778	CMH[M + Na]$^+$	d18:1	C20:0	1157	CTH[M + Na]$^+$	d18:1	C24:1
788	SM[M + H]$^+$	d18:0	C22:0	1159	CTH[M + Na]$^+$	d18:1	C24:0
810	SM[M + Na]$^+$	d18:1	C22:0				
824	SM[M + Na]$^+$	d18:1	C23:0				
834	CMH[M + Na]$^+$	d18:1	C24:0				

SM, sphingomyelin; CMH, glucosylceramide; CTH, globotriaosylceramide.

Human Platelets

About 50 mg of packed platelets washed with saline are shaken vigorously with 10 ml of chloroform–methanol (2:1, v/v) using a vortex mixer. After centrifugation, the supernatant is decanted and the precipitate is extracted with 5 ml of the same solution. The lipid extracts are combined and evaporated under N_2. The residue is treated with about 1 ml of acetone to remove cholesterol and simple lipids. After decanting, the residue is again extracted with about 1 ml of diethyl ether to remove glycerophospholipids. After decanting again, the residue is treated with 1 ml of 0.5 *N* NaOH in methanol at 50° for 1 hr.

After the saponification, the reaction mixture is acidified with one drop of 6 *N* HCl together with one drop of distilled water and shaken vigorously with 1 ml of hexane using a vortex mixer. After the upper phase containing free fatty acids is removed, the lower phase is again treated with hexane. After this procedure is repeated, the lower phase is evaporated under N_2 and the residue is shaken vigorously with 1 ml of chloroform–methanol–water (86:14:1, v/v; theoretical lower phase) and 1 ml of chloroform–methanol–water (3:48:47, v/v; theoretical upper phase) to remove salts. The upper phase and the lower phase are separated by centrifugation at 3000 rpm for 10 min. After the upper phase is carefully removed with a micropipette, the lower phase is evaporated under N_2. This residue is rextracted to remove salts, and the final residue is dissolved with 20 μl of chloroform–methanol (1:1, v/v) with a microsyringe.

One μl of the solution is mixed vigorously with 1 μl of the matrix solution of 2,5-DHB in an Eppendorf tube and centrifuged. One microliter of the supernatant is applied to the sample plate. As described above, it is subjected to DE MALDI-TOF MS analysis by the same procedure. The mass spectrum displayed molecular weight-related ions corresponding to individual molecular species of sphingomyelin, glucosylceramide, lactosylceramide, globotriaosylceramide, globotetraosylceramide, and GM2 at the wide mass range in the positive ion mode, as identified in Fig. 8. Judging by the counts in the mass spectrum, sphingomyelin was found to be a major sphingolipid and other minor multiple glycosphingolipids were definitely confirmed.

Necrotic Tissues from Head of Femur of a Patient with Fabry Disease and a Control Subject

About 100 mg of wet necrotic tissue from the head of a femur of two patients with or without Fabry disease separately was homogenized vigorously with 10 ml of chloroform–methanol (2:1, v/v) with a pestle in a mortar. After the homogenate was filtered, the total lipid extract (8 ml)

was mixed vigorously with 2 ml of distilled water and partitioned into an upper phase and a lower phase by centrifugation. After the upper phase was removed carefully, the lower phase was evaporated under N_2. The residue was treated to obtain the alkali-stable lipid fraction as described above. The solution was subjected to the DE MALDI-TOF MS analysis by the same procedure.

As shown in Fig. 9, the mass spectrum of the patient with Fabry disease certainly suggested the molecular weight-related ions corresponding to accumulated globotriaosylceramides. All molecular weight-related ions of the globotriaosylceramide and other sphingolipids in the alkali-stable lipid fraction of the patient were identified as shown in Fig. 10.

These examples illustrate the utility of this method with impure as well as purified sphingolipids. It is necessary to confirm the identities of the peaks by another method (such as MS/MS); however, once this has been done, DE MALDI-TOF MS is a simple and convenient analytical procedure.

[9] Purification of Sphingolipid Classes by Solid-Phase Extraction with Aminopropyl and Weak Cation Exchanger Cartridges

By JACQUES BODENNEC, GÉRARD BRICHON, GEORGES ZWINGELSTEIN, and JACQUES PORTOUKALIAN

Introduction

Isolation and purification of molecules from complex lipid mixtures is generally done by preparative thin-layer chromatography (TLC),[1] liquid chromatography on different silica-based sorbents,[2] and high-performance liquid chromatography (HPLC).[3] However, TLC can be cumbersome and time consuming. Silicic acid column chromatography often requires considerable amounts of solvents and larger amounts of samples. Isolation of

[1] B. Fried, *in* "Thin-Layer Chromatography" (B. Fried and J. Sherma, eds.), Vol. 81, p. 277. Wiley, New York, 1984.

[2] J. B. Davenport, *in* "Biochemistry and Methodology of Lipids" (A. R. Johnson and J. B. Davenport, eds.), p. 151. Wiley-Interscience, New York, 1971.

[3] W. W. Christie, *in* "Height-Performance Liquid Chromatography and Lipids. A Practical Guide," p. 87. Pergamon Press, Oxford, 1987.

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