methods

Characterization and direct quantitation of sphingoid base-1-phosphates from lipid extracts: a shotgun lipidomics approach

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Abstract **Here, we have extended shotgun lipidomics for the characterization and quantitation of sphingosine-1-phosphate (S1P) and dihydrosphingosine-1-phosphate (DHS1P) in crude lipid extracts in the presence of ammonium hydroxide by using precursor ion scanning of *m/z* 79.0 (corresponding to $[PO_3]^-$) in the negative-ion mode. It is demonstrated that a broad linear dynamic range for the quantitation of both S1P and DHS1P and a detection limit at low amol/μl concentration are achieved using this approach. The developed method for the quantitation of sphingoid base-1-phosphates is generally simpler and more efficient than other previously published methods. Multiple factors influencing the quantitation of sphingoid base-1-phosphates, including ion suppression, extraction efficiency, and potential overlapping with other molecular species, were examined extensively and/or are discussed. Mass levels of S1P and DHS1P in multiple biological samples, including human plasma, mouse plasma, and mouse brain tissues (e.g., cortex, cerebellum, spinal cord, and brain stem), were determined by the developed methodology. Accordingly, this technique, as a new addition to shotgun lipidomics technology, will be extremely useful for understanding the pathways of sphingolipid metabolism and for exploring the important roles of sphingoid base-1-phosphates in a wide range of physiological and pathological studies.**—Jiang, X., and X. Han. **Characterization and direct quantitation of sphingoid base-1-phosphates from lipid extracts: a shotgun lipidomics approach.** ***J. Lipid Res.*** **2006.** 47: **1865–1873.**

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Sphingoid base-1-phosphates mainly include two types of compounds: sphingosine-1-phosphate (S1P) and dihydrosphingosine-1-phosphate [or sphinganine-1-phosphate (DHS1P)]. S1P is normally present in low abundance (~0.1 mol% of total cellular lipids under normal physiological conditions), and its intracellular levels are under strict regulation by the enzymes that control S1P biosynthesis and degradation (1–7). S1P is thought to be produced exclusively by the phosphorylation of sphingosine as catalyzed by sphingosine kinase, whereas its degradation occurs either through hydrolytic cleavage to produce palmitaldehyde and phosphoethanolamine or by dephosphorylation to sphingosine (1–7). DHS1P undergoes a similar path of biosynthesis and metabolism to that of S1P (4).

S1P is an essential bioactive sphingolipid metabolite that has recently become the focus of intense interest. S1P functions are mediated by its binding to a family of five G protein-coupled receptors (i.e., $S1P_1$–$S1P_5$). S1P receptors are widely expressed and are thought to regulate important physiological actions (e.g., neuron survival, immunity, smooth muscle contraction, vascular development, control of vascular tone, cardiac function, cardiac development, and vascular permeability) (3, 5, 7, 8). In addition, S1P may participate in various pathological conditions (e.g., autoimmunity, transplant rejection, cancer, angiogenesis, female infertility, and myocardial infarction) (4, 5, 9). S1P also serves as a second messenger that is important for the regulation of calcium homeostasis, cell growth, and suppression of apoptosis (1, 3–12). DHS1P has been found to bind to S1P receptors as S1P, but its binding ability is less potent than that of S1P (13). Moreover, DHS1P stimulates the expression of matrix metalloproteinase 1, which is associated with several pathological processes, including tumor growth and metastasis (14). However, the role(s) of DHS1P in biological processes is not well defined.

To investigate and define the important biological functions and role(s) of S1P and DHS1P, it is essential to have a simple, sensitive, and reliable methodology for the simultaneous quantification of the mass contents of these low-abundant sphingoid base-1-phosphates in biological

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samples. Multiple methodologies have recently been developed and used for the quantification of S1P and DHS1P present in biological samples. These methods include a radioreceptor binding assay (15), enzymatic measurement (16, 17), quantification after chemical modification (18), and determination by HPLC (19–24), MS (25), or liquid chromatography tandem mass spectrometry (LC-MS/MS) (26–31).

The radioreceptor binding assay involves competitive replacement of [^{3}H]S1P with S1P from the $S1P_1$ receptor (15). In the enzymatic method, S1P is hydrolyzed to sphingosine by alkaline phosphatase, followed by recombinant sphingosine kinase-catalyzed phosphorylation with [γ-^{32}P]ATP to form [^{32}P]S1P (16, 17). The chemical modification method involves acetylation of S1P with [^{3}H]acetic anhydride to its *N*-[^{3}H]acetylated derivative (18). Liquid scintillation spectroscopy is used to quantify the radiolabeled S1P mass content in each of these three methods. The drawbacks of these methods include the use of radioactive reagents, the labor-intensiveness of the procedures, and the lack of structural information.

In the HPLC approach, S1P/DHS1P are either directly converted to fluorescent *o*-phthaladehyde derivatives (19–21, 24) or dephosphorylated with alkaline phosphatase followed by conversion to either fluorescent *o*-phthaladehyde (22) or *o*-naphthalene-2,3-dicarboxyaldehyde derivatives (23). The fluorescent derivatives are then separated by HPLC and detected by a spectrofluorometer. In addition to laborious chemical modification, the HPLC method does not adequately provide structural details regarding S1P and DHS1P molecular species. S1P has also been derivatized to its *N*-phenylthiocarbamate and detected by fast-atom bombardment MS (25). This method does not allow reliable quantification of sphingoid base-1-phosphates present in biological samples, because DHS1P is used as an internal standard and most of the biological samples contain various amounts of DHS1P. Unmodified S1P has been quantified successfully by LC-MS/MS using a multiple-reaction monitoring technique in the positive-ion mode (26–30). However, the broad peak on LC-MS/MS analysis (26) and sample carryover (30, 31) are apparent disadvantages of this method. To overcome these drawbacks, S1P and DHS1P have been converted to their bisacetylated derivatives and subsequently analyzed by negative-ion LC-MS/MS (31).

Recently, we developed an approach to globally analyze individual lipid molecular species of a biological sample directly from a lipid extract, now known as shotgun lipidomics (32–35). Shotgun lipidomics is a multistep process using multiplexed extractions, intrasource separation (36), and multidimensional mass spectrometry and array analysis (35). Here, we have developed a simple, rapid, and sensitive technique for the quantitation of S1P and DHS1P directly from chloroform extracts of biological samples by electrospray ionization (ESI)-MS/MS (precursor ion scan) in the negative-ion mode, thereby extending shotgun lipidomics to the identification and quantification of sphingoid base-1-phosphate molecular species. In this study, multiple factors that might affect the quantification of S1P and DHS1P, including limitation of detection, linear dynamic range, ion suppression, extraction efficiency, and potential overlapping with other molecular species, were examined extensively and are discussed. Mass levels of S1P and DHS1P in multiple biological samples, including mouse plasma and brain tissues (e.g., cortex, cerebellum, spinal cord, and brain stem), were determined by the developed methodology. We anticipate that this technique, as a new addition to shotgun lipidomics technology, will be very useful for understanding the interrelationships between sphingolipid metabolism and the important roles of sphingoid base-1-phosphates in biological processes in a variety of physiological and pathological contexts.

MATERIALS AND METHODS

Materials

Synthetic S1P, C17 S1P (a 17 carbon analog of S1P), and DHS1P were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL). The purity of all sphingoid base-1-phosphates was examined by ESI-MS before use. All solvents used for sample preparation and for mass spectrometric analysis were obtained from Burdick and Jackson (Muskegon, MI). Concentrated ammonium hydroxide (28–30%) was purchased from Fisher Scientific (Pittsburgh, PA). The S1P, C17 S1P, and DHS1P stock solutions were made in chloroform-methanol (1:1, v/v) and stored under nitrogen at −20°C.

Preparation of lipid extracts from biological samples

Human plasma was collected from healthy individuals at 65 ± 5 years of age. Male mice (C57BL/6; 4 months of age) were purchased from the Jackson Laboratory (Bar Harbor, ME). Mice were euthanized by asphyxiation with carbon dioxide. Blood plasma from each mouse was separately harvested immediately. Brain tissues were dissected, quickly dried, and immediately freeze-clamped at the temperature of liquid nitrogen. Wafers were pulverized into a fine powder with a stainless-steel mortar and pestle. Protein assays on the fine powders were performed using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL) with BSA as the standard. The lipid content was normalized to the protein content and quantified directly.

Fine powders of each individual brain tissue sample (~10 mg) were weighed into a disposable glass culture test tube, and C17 S1P (50 pmol/mg protein; used as an internal standard for the quantitation of sphingoid base-1-phosphates) was added. The tissues were homogenized and extracted three times with 2 ml of 2:1 chloroform-methanol containing 5% acetic acid. The combined crude extracts were dried under a nitrogen stream. Each individual residue was then resuspended in 4 ml of chloroform-methanol (1:1) and reextracted against 1.8 ml of 5% acetic acid aqueous solution, and the chloroform extract was dried under a nitrogen stream. Each individual residue was resuspended in 1 ml of chloroform and filtered with a 0.2 μm polytetrafluoroethylene syringe filter into a 10 ml glass centrifuge tube. This procedure was repeated at least four times to maximize the transfer of lipids. The chloroform solution was subsequently dried under a nitrogen stream, and each individual residue was reconstituted with a volume of 1 ml/mg protein (based on the original protein content of the samples) in 1:1 chloroform-methanol. The lipid extracts were finally flushed with nitrogen, capped, and stored at −20°C for ESI-MS analysis (typically ana-

lyzed within 1 week). Each lipid solution was further diluted ~10-fold with chloroform-methanol (1:1, v/v) immediately before direct infusion and lipid analysis by ESI-MS. A small volume of concentrated ammonium hydroxide (2%, v/v) was added to the diluted lipid solutions immediately before further lipid analyses in the negative-ion mode.

To extract lipids from plasma samples, an aliquot (0.1 ml) from each individual or animal plasma sample was transferred to a clean glass tube, and C17 S1P internal standard (1 nmol/ml) was added. The plasma lipids were extracted according to the procedure for lipid extraction of brain tissues except for omitting the extraction step with 2 ml of 2:1 chloroform-methanol containing 5% acetic acid three times. In addition, a lipid extract from mouse brain cortex was also prepared similarly except that 5% acetic acid was replaced by 10 mM LiCl in the aqueous phase. Each mixture of S1P, DHS1P, and C17 S1P was extracted twice using the Bligh and Dyer method in the presence of 5% acetic acid in the aqueous phase before analysis.

ESI-MS/MS analysis of sphingoid base-1-phosphates

A triple-quadrupole mass spectrometer (ThermoElectron TSQ Quantum Ultra, San Jose, CA) equipped with an electrospray ion source and operating under an Xcalibur software system was used as described previously (33, 37). The spray voltage was maintained at 3 kV in the negative-ion mode. An offset voltage on the ion transfer capillary was set to −17 V. The heater temperature along the ion transfer capillary was maintained at 250°C. The sheath gas (nitrogen) pressure was 2 p.s.i. The diluted lipid extract solution was infused directly into the ESI source at a flow rate of 4 μl/min with a syringe pump. Typically, a 1 min period of signal averaging from 0.2 s per scanning in the profile mode was used for each MS spectrum. For MS/MS, the collision gas (argon) pressure was set at 1.0 mTorr, and a collision energy of 24 eV in the product ion mode and precursor ion mode was used for all sphingoid base-1-phosphates. Ratiometric comparison of peak intensities from precursor ion scanning of *m/z* 79.0 (corresponding to $[PO_3]^-$) was used for the identification and quantification of sphingoid base-1-phosphates. Typically, a 2 min period of signal averaging from 0.2 s per scanning in the profile mode was used for each MS/MS spectrum.

RESULTS AND DISCUSSION

ESI-MS analysis of sphingoid base-1-phosphate molecular species in both negative- and positive-ion modes

Sphingoid base-1-phosphate molecular species are unique, carrying a monoesterified phosphate moiety and a primary amine in each individual molecular species. Therefore, they are weakly zwitterionic and their electrical properties can vary from anionic under alkaline conditions to cationic under acidic conditions. According to the theory of intrasource separation, these types of compounds can be ionized in both the positive- and negative-ion modes of an ESI ion source under suitable experimental conditions.

ESI-MS analyses of an equimolar mixture of S1P, DHS1P, and C17 S1P (varied from 1 pmol/μl each to 1 amol/μl each) in the negative-ion mode in the presence of 2% concentrated ammonium hydroxide in chloroform-methanol (1:1, v/v) demonstrate three essentially equally abundant peaks of deprotonated sphingoid base-1-phosphates (left panel of **Fig. 1**). These results indicate that a detection sensitivity of 1 amol/μl for the analysis of sphingoid base-1-phosphate molecular species in the negative-ion mode under experimental conditions could be readily achieved (spectrum a4 in Fig. 1). Moreover, the ionization response factors for these species under experimental conditions are essentially identical.

Similarly, positive-ion ESI-MS analyses of this identical mixture of sphingoid base-1-phosphates in the presence of 0.1% formic acid in chloroform-methanol (1:1, v/v) show three essentially equally intense peaks of protonated sphingoid base-1-phosphates (spectra not shown). However, the detection limitation (~1 fmol/μl) of these molecular species in the positive-ion mode under experimental conditions is much higher than that obtained in the negative-ion mode. The identities of these molecular ions in both the negative- and positive-ion modes were confirmed by product ion analyses as shown below.

Characterization of sphingoid base-1-phosphates by ESI-MS/MS in both negative- and positive-ion modes

Product ion ESI-MS analysis of the C17 S1P ion at *m/z* 364.2 [as shown in the mass spectrum of the equimolar mixture of sphingoid base-1-phosphates (Fig. 1)] in the negative-ion mode shows a very abundant characteristic product ion at *m/z* 79.0 (**Fig. 2A**), corresponding to $[PO_3]^-$, as described previously (38). In addition, a low-abundant fragment ion at *m/z* 97.0, corresponding to $[H_2PO_4]^-$, is also present in the product ion mass spectrum (Fig. 2A). Product ion ESI-MS analyses of both S1P and DHS1P show identical fragmentation patterns to that of C17 S1P (Fig. 2).

Because the fragment ion at *m/z* 79.0 is present in the collision-induced dissociation of all examined sphingoid base-1-phosphates and is present as the most abundant fragment ion, precursor ion scanning of *m/z* 79.0 in the negative-ion mode was performed to analyze equimolar mixtures of sphingoid base-1-phosphate molecular species at different concentrations. The analyses demonstrate three molecular ion peaks of essentially equal intensity corresponding to the analyzed sphingoid base-1-phosphates (right panel of Fig. 1). Although ESI-MS analysis shows a somewhat noisy mass spectrum at a concentration of 1 amol/μl for each sphingoid base-1-phosphate molecular species (spectrum a4 in Fig. 1), precursor ion analysis under optimal collision-induced dissociation conditions demonstrates that the detection limit for the analysis of sphingoid base-1-phosphate molecular species under the experimental conditions used is much lower than 1 amol/μl (spectrum b4 of Fig. 1). The optimal fragmentation conditions were determined to be 24 eV for the collision energy and 1 mTorr for the collision gas pressure (i.e., argon).

Collision-induced dissociations of the protonated C17 S1P and S1P yield abundant product ions at *m/z* 250.2 and 264.2, respectively (**Fig. 3A, B**), representing neutral loss of H_3PO_4 and water from their corresponding molecular ions (**Scheme 1**). The resultant allylic cation is stabilized by an adjacent enamine (Scheme 1). Product ion ESI-MS analysis of DHS1P in the positive-ion mode shows two

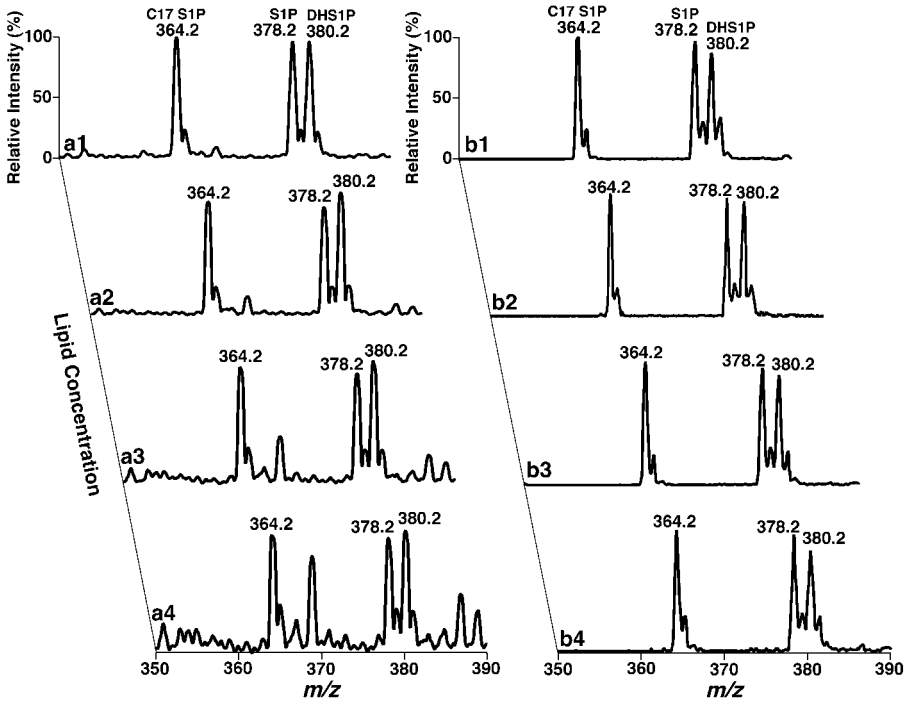


Fig. 1. Negative-ion electrospray ionization mass spectrometry (ESI-MS) and ESI-tandem mass spectrometry (MS/MS) analyses of equimolar mixtures of sphingoid base-1-phosphates. Equimolar mixtures of sphingosine-1-phosphate (S1P), C17 S1P (a 17 carbon analog of S1P), and dihydrosphingosine-1-phosphate (DHS1P) were prepared and extracted in the presence of 5% acetic acid. Negative-ion ESI-MS analyses (left) and MS/MS analyses in the precursor ion mode by monitoring *m/z* 79.0 (right) were performed in the presence of 2% concentrated ammonium hydroxide, as described in Materials and Methods, at concentrations of 1 pmol/μl each (spectra a1 and b1), 10 fmol/μl each (spectra a2 and b2), 0.1 fmol/μl each (spectra a3 and b3), and 1 amol/μl each (spectra a4 and b4). A 2 min period of signal averaging 0.2 s per scanning in the profile mode was used for each MS/MS spectrum. Unlabeled ion peaks are instrument artifacts. The mass resolution setting for these analyses is full width at half-maximum height (FHMW) 0.6 Th.

abundant product ions at *m/z* 284.2 and 266.2 (Fig. 3C). The ion at *m/z* 284.2 likely results from the neutral loss of H_3PO_4, which may further lose a water molecule to give rise to the ion at *m/z* 266.2 (Scheme 1). Alternatively, the ion at *m/z* 266.2 is produced directly by the loss of H_3PO_4 and water (Scheme 1). As anticipated, MS/MS analysis by neutral loss of 116.0 u (corresponding to a loss of H_3PO_4 and water) from an equimolar mixture of sphingoid base-1-phosphates shows two peaks of essentially equal intensity of the protonated C17 S1P and S1P. However, the ion peak intensity of the protonated DHS1P is much lower compared with those of protonated C17 S1P and S1P (spectra not shown) as a result of the presence of different fragmentation pathways. These results indicate that neutral loss of 116.0 u in the positive-ion mode is not quite suitable for the quantitation of sphingoid base-1-phosphate molecular species.

Quantitation of sphingoid base-1-phosphate molecular species by precursor ion scanning of *m/z* 79.0 in the negative-ion mode

MS/MS analyses of equimolar mixtures of C17 S1P, S1P, and DHS1P through precursor ion scanning of *m/z* 79.0 show quantitative ion peak intensities in a broad range of concentrations (right panel of Fig. 1). Further examination of a variety of different ratios of S1P or DHS1P to C17 S1P at different concentrations was also performed. As anticipated, a quantitative relationship of the ion peak intensity ratio versus the molar ratio of C17 S1P to S1P is present, indicating that the presence of one methylene difference between C17 S1P and S1P does not apparently affect the quantitation of S1P (using C17 S1P as an internal standard) through precursor ion scanning of *m/z* 79.0 in the negative-ion mode.

Notably, a statistically lower ion peak intensity of DHS1P relative to those of C17 S1P and S1P [after correction for the effect of M+2 ^{13}C S1P isotopomer on the peak intensity of DHS1P, as described previously (39)] is observed consistently. For example, **Fig. 4** shows the relationships between the molar ratios and peak intensity ratios of S1P or DHS1P to C17 S1P at a fixed concentration of 0.1 pmol/μl. S1P and DHS1P to C17 S1P show linear correlation coefficients (γ^2) of 0.9992 and 0.9977 and intercepts close to zero (i.e., 0.03 and −0.054), respectively. However, a lower slope of 0.920 for DHS1P versus C17 S1P was observed compared with that for S1P versus C17 S1P (i.e., 1.001). This is likely attributable to the presence of differential fragmentation pathway(s) of DHS1P compared with C17 S1P and S1P. For example, a minor fragment ion at *m/z* 256.2 is present in the product ion mass spectrum of

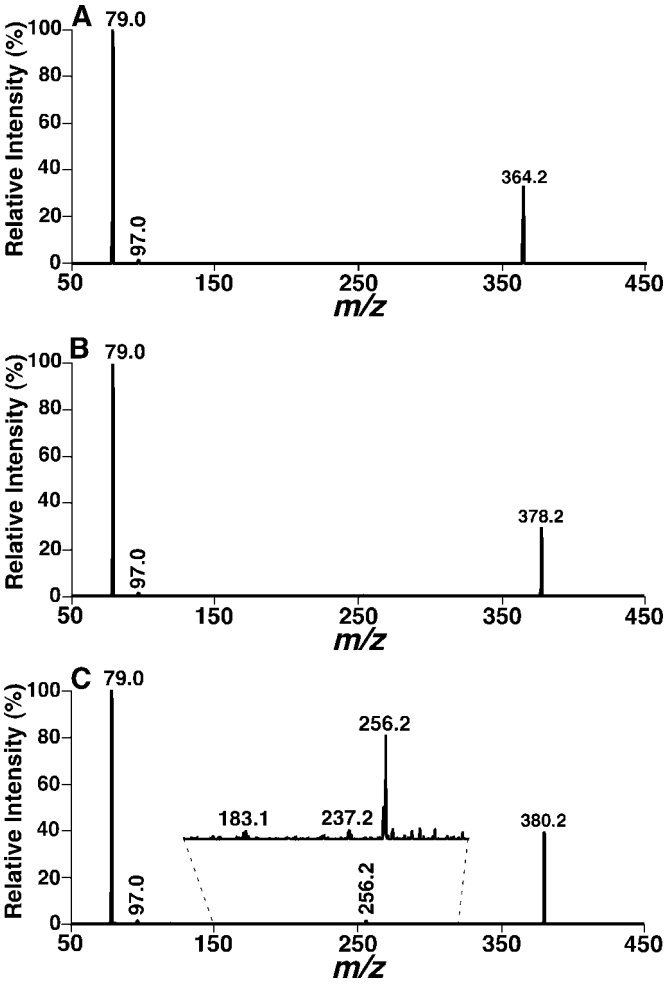


Fig. 2. Product ion characterization of C17 S1P (A), S1P (B), and DHS1P (C) in the negative-ion mode. Negative-ion ESI-MS analyses of sphingoid base-1-phosphates were performed as described in the legend of Fig. 1. Product ion analysis during collision-induced dissociation of each individual deprotonated sphingoid base-1-phosphate molecular species was performed by selecting an ion of interest in the first quadrupole and analyzing the fragments in the third quadrupole while collisional activation was performed in the second quadrupole. A collision gas pressure of 1.0 mTorr and a collision energy of 24 eV were used in the analysis.

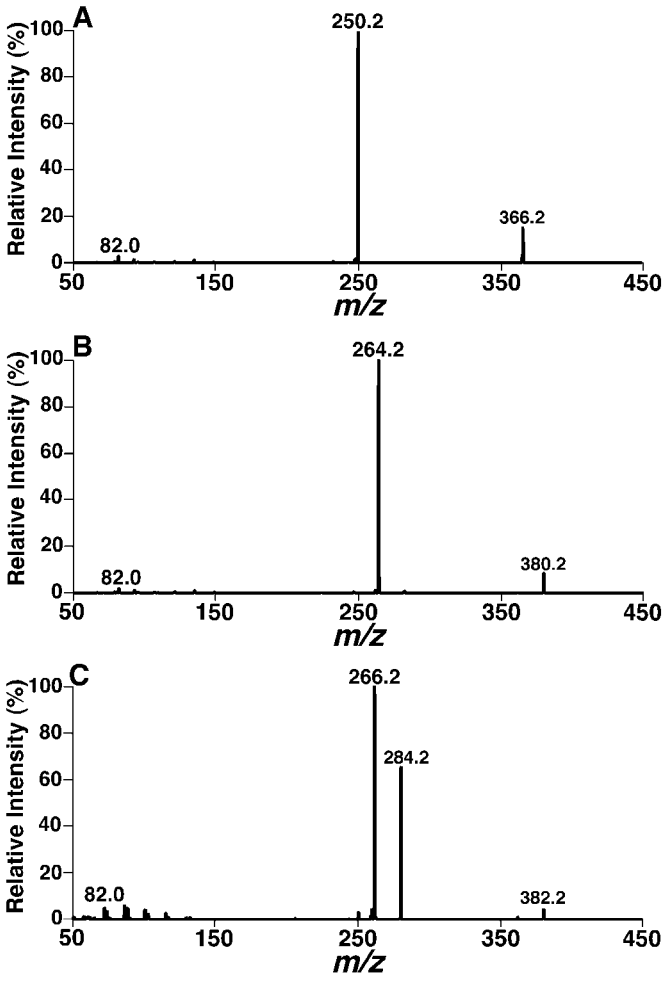
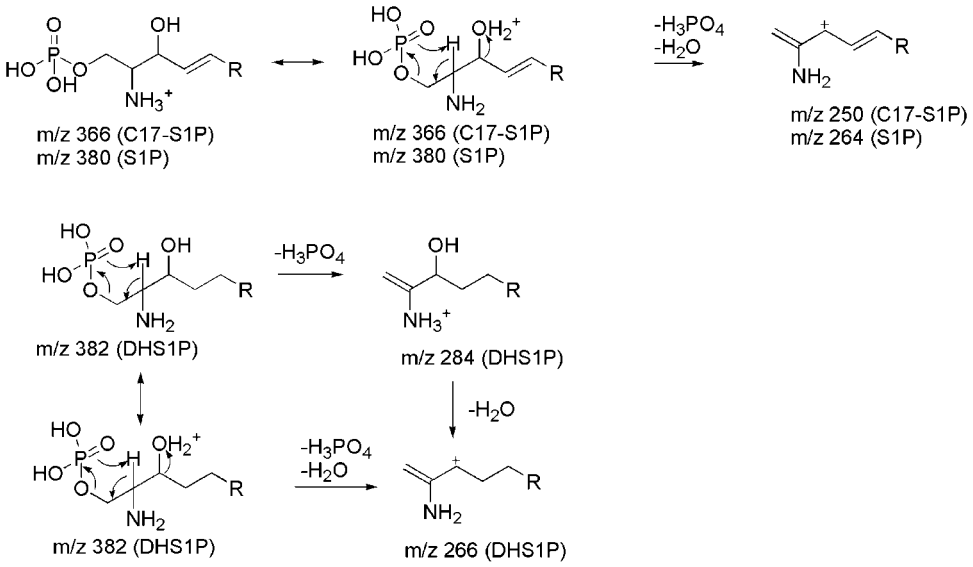


Fig. 3. Product ion characterization of C17 S1P (A), S1P (B), and DHS1P (C) in the positive-ion mode. Positive-ion ESI-MS analyses of an equimolar mixture of sphingoid base-1-phosphates (1 pmol/μl each) in the presence of 0.1% formic acid demonstrate three equally intense, protonated molecular ions. Product ion analysis during collision-induced dissociation of each individual protonated sphingoid base-1-phosphate molecular species was performed by selecting an ion of interest in the first quadrupole and analyzing the fragments in the third quadrupole while collision activation was performed in the second quadrupole. A collision gas pressure of 1.0 mTorr and a collision energy of 24 eV were used in the analyses.

DHS1P but not in those of C17 S1P and S1P (Fig. 2). Therefore, a correction factor of 0.92 must be applied for the quantitation of DHS1P when C17 S1P is used as an internal standard.

Next, multiple factors that might influence the quantitation of sphingoid base-1-phosphates were examined. We first examined the extraction efficiency of the sphingoid base-1-phosphates, because these compounds are not generally soluble in most organic solvents as a result of their high hydrophilicity, thereby complicating their quantitation. After examining several of the previously used extraction methods with an organic phase of chloroform-methanol against neutral, basic, and acidic aqueous phases, it was found that extraction under acidic conditions with 5% (v/v) acetic acid gave the best extraction efficiency of the examined sphingoid base-1-phosphates, whereas they were almost completely lost under neutral and basic conditions. However, it should be pointed out that to avoid the effects of residual acetic acid on the efficient ionization of sphingoid base-1-phosphates in the negative-ion mode, an excessive amount of ammonium hydroxide [i.e., 2% (v/v) concentrated ammonium hydroxide] in 1:1 (v/v) chloroform-methanol was used in the ESI-MS analysis of sphingoid base-1-phosphates. In addition, we also examined the recovery of sphingoid base-1-phosphates by the described extraction method as follows. Known amounts of S1P and DHS1P were extracted in the presence of 5% acetic acid in the aqueous phase, and a known amount of C17 S1P was added to each extract as a standard to quantitate the amount of S1P and DHS1P present in the extract. Recoveries of 58, 77, and 98% were



Scheme 1. Proposed fragmentation pathways of protonated sphingosine-1-phosphate (S1P) and dihydrosphingosine-1-phosphate (DHS1P) in the positive-ion mode.

determined from 25, 50, or 100 pmol of total sphingoid base-1-phosphates in each sample, respectively. It should be noted that performing additional extractions from a given sample could always maximize the extraction recovery. Moreover, an incomplete extraction will not affect the quantitation using the developed method owing to the presence of an internal standard (i.e., C17 S1P), provided that the mass contents of sphingoid base-1-phosphates in an extract are in excess of the instrument detection limit.

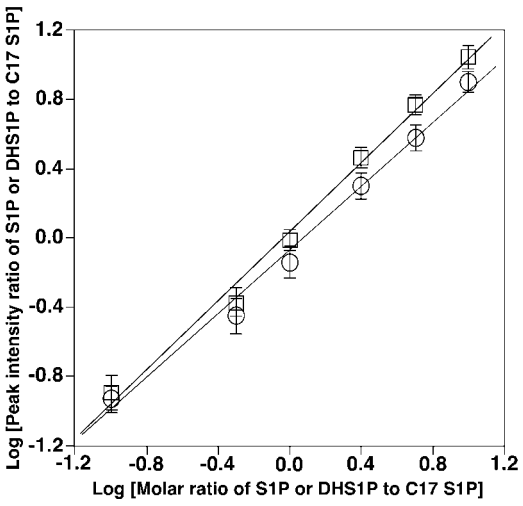


Fig. 4. Correlation of the molar ratios with the ion peak intensity ratios of S1P or DHS1P to C17 S1P. Mixtures composed of different molar ratios of S1P (open squares) or DHS1P (open circles) to C17 S1P (the concentration of C17 S1P was fixed at 0.1 pmol/μl) were prepared separately. Negative-ion MS/MS analyses through precursor ion scanning of *m/z* 79.0 in the presence of 2% concentrated ammonium hydroxide were performed through scanning the first quadrupole in the mass range of interest and monitoring the third quadruple for the ion at *m/z* 79.0 while collisional activation was performed in the second quadrupole. The collision gas pressure was 1.0 mTorr, and a collision energy of 24 eV was used in the analyses. The data points represent means ± SEM of the peak intensity ratios from four separate mixture preparations of each molar ratio of S1P or DHS1P to C17 S1P.

The effects of the presence of other abundant lipids on sphingoid base-1-phosphate (i.e., so-called ion suppression) were also examined. We added 0.1 mol% of C17 S1P to a total lipid extract of mouse brain cortex, which was extracted under neutral conditions in the presence of 10 mM LiCl and in which only minimal amounts of endogenous sphingoid base-1-phosphates were present (see below). After adding various amounts of S1P and DHS1P to this lipid extract containing 0.1% C17 S1P, the lipid solution was diluted to a mass level of 0.1 pmol C17 S1P/μl (i.e., total lipid mass content at 100 pmol/μl) and MS and MS/MS analyses were performed. **Figure 5** shows the typical results from such an experiment. Spectrum A represents a mass spectrum in the region of interest without the addition of sphingoid base-1-phosphates, and the inset of spectrum A shows its precursor ion spectrum by monitoring at *m/z* 79.0. The spectrum in the inset is displayed after normalizing the precursor ion spectrum to spectrum B, which was acquired by precursor ion scanning of *m/z* 79.0 in the presence of 0.1, 0.01, and 0.01 pmol/μl C17 S1P, S1P, and DHS1P, respectively (Fig. 5). Analyses of different ratios of S1P and DHS1P to C17 S1P, which was fixed at 0.1 mol% of total mouse cortex lipids, demonstrate essentially identical linear relationships between the molar ratios and the peak intensity ratios of S1P or DHS1P to C17 S1P and those obtained from the absence of other abundant lipids, as shown in Fig. 4. These results indicate that the presence of other abundant lipids does not interfere with the accurate quantitation of sphingoid base-1-phosphates (i.e., shotgun lipidomics of these compounds can be performed successfully).

Finally, because no chromatographic separation is performed in shotgun lipidomics and the $[PO_3]^-$ fragment ion is not specific to sphingoid base-1-phosphates, it is im-

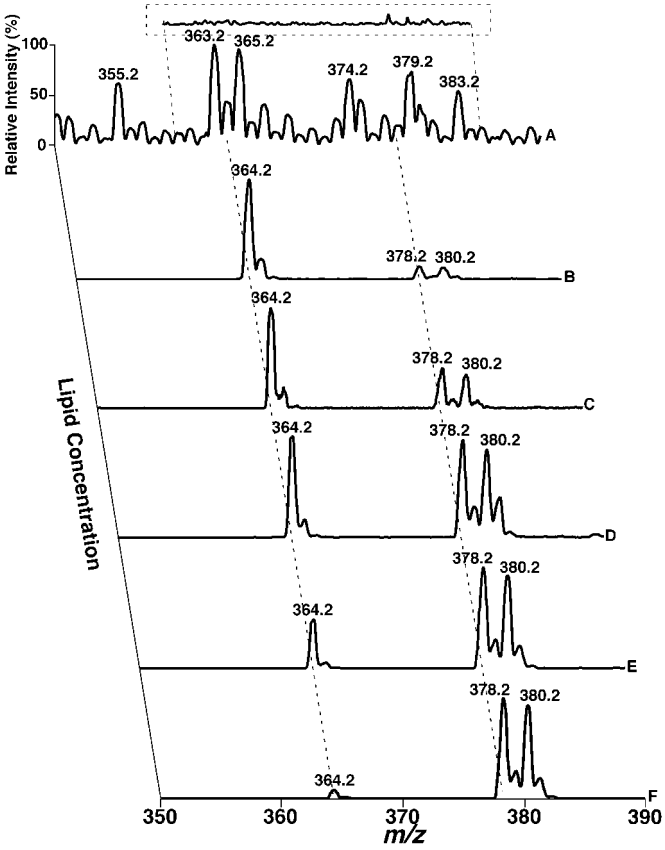


Fig. 5. Effects of the presence of other abundant lipids on the quantitation of sphingoid base-1-phosphates through precursor ion scanning of *m/z* 79.0 from a lipid extract in the negative-ion mode. Lipids of mouse brain cortex were extracted by a modified procedure of Bligh and Dyer in the presence of 10 mM LiCl. A fixed amount of C17 S1P (0.1 mol% of total lipids) and various molar ratios of S1P and DHS1P at equimolar concentrations to C17 S1P were added to the mouse cortex lipid extract. The lipid solution was diluted to a concentration of 100 pmol/μl total lipids (i.e., 0.1 pmol/μl C17 S1P) with 1:1 chloroform-methanol containing 2% concentrated ammonium hydroxide before direct infusion to the ESI ion source. The endogenous mass contents of sphingoid base-1-phosphates are negligible compared with the amounts of exogenously added sphingoid base-1-phosphates, as discussed in the text. ESI-MS analysis of brain cortical lipids without the addition of exogenous sphingoid base-1-phosphates was performed in the negative-ion mode (spectrum A). The precursor ion ESI-MS spectrum of sphingoid base-1-phosphates in the brain cortical lipid extract through precursor ion scanning of *m/z* 79.0 is shown in the inset of spectrum A, as displayed after normalization of the ion current to the ion peak of C17 S1P in spectrum B. ESI-MS analyses of sphingoid base-1-phosphates through precursor ion scanning of *m/z* 79.0 demonstrate a linear correlation between the molar ratios of S1P or DHS1P to C17 S1P (i.e., 0.01, 0.05, 0.1, 0.5, and 1 pmol/μl vs. 0.1 pmol/μl) and their peak intensity ratios (0.1, 0.5, 1, 5, and 10 in spectra B, C, D, E, and F, respectively). The mass resolution setting for these analyses is FHMW 0.6 Th. A 2 min period of signal averaging 0.2 s per scanning in the profile mode was used for each MS/MS spectrum.

portant to determine potential interferences from other components in biological samples using precursor ion scanning of *m/z* 79.0 (i.e., $[PO_3]^-$) in the negative-ion mode. In theory, any components containing a phosphate group might produce $[PO_3]^-$ during collision-induced dissociation and interfere with quantitation. In practice, although precursor ion scanning of *m/z* 79.0 in the negative-ion mode of a mixture containing C17 S1P, myristoryl lysophosphatidic acid (*m/z* 381.2, a common internal standard for the analysis of lysophosphatidic acid), and dimyristoryl phosphatidic acid (*m/z* 591.4, a common internal standard for the quantitation of phosphatidic acid) displays all of the species in the sample (spectrum not shown), no overlaps from these species and other recognizable lipid molecular species containing a phosphate group and possessing an identical *m/z* to C17 S1P, S1P, and DHS1P in mammalian samples are present.

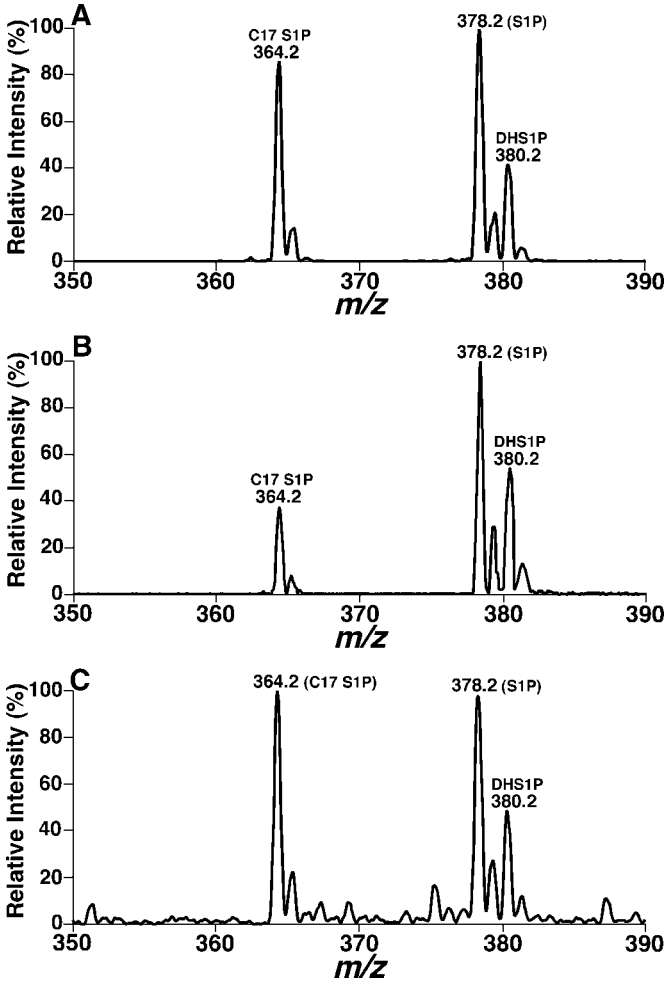


Fig. 6. MS/MS spectra of sphingoid base-1-phosphates in lipid extracts of mouse plasma (A), spinal cord (B), and cerebellum (C) through precursor ion scanning of *m/z* 79.0. Total lipids of mouse plasma, spinal cord, and cerebellum were extracted using a modified procedure of Bligh and Dyer in the presence of 5% acetic acid in the aqueous phase, as described in Materials and Methods. MS/MS spectra of sphingoid base-1-phosphates in lipid extracts of mouse plasma, spinal cord, and cerebellum were acquired through scanning the first quadrupole in the mass range of interest and monitoring the third quadruple for the ion at *m/z* 79.0 while collision activation was performed in the second quadrupole. The collision gas pressure was set at 1.0 mTorr, and a collision energy of 24 eV was used in the analyses. A 2 min period of signal averaging 0.2 s per scanning in the profile mode was used for each MS/MS spectrum.

However, we cannot exclude the possibility that no other species could interfere with the quantitation of sphingoid base-1-phosphates from other sample sources (e.g., plants, bacteria, etc.) by shotgun lipidomics.

Quantitation of S1P and DHS1P in lipid extracts of various biological samples

The developed shotgun lipidomics approach was used to directly quantitate S1P and DHS1P mass contents in lipid extracts of human plasma, mouse plasma, and mouse spinal cord and brain tissues (including cortex, cerebellum, and brain stem). The averaged mass contents of S1P and DHS1P in lipid extracts from human plasma of seven healthy individuals (65 ± 5 years of age) are 703 ± 41 and 327 ± 34 pmol/ml, respectively. These results are in good agreement with those from other studies using different methods (31). **Figure 6** shows a series of typical precursor ion ESI-MS/MS spectra of sphingoid base-1-phosphates in lipid extracts of mouse plasma, spinal cord, and cerebellum. Quantitative results of S1P and DHS1P in the lipid extracts of mouse samples are summarized in **Table 1**.

To the best of our knowledge, the results described here reporting the presence of both S1P and DHS1P in mouse tissues represent the first documentation of S1P and DHS1P in these tissues. Moreover, considerable amounts of S1P and DHS1P (Table 1) were found in mouse plasma, and these results may reflect the release of sphingoid base-1-phosphates from platelets, an important reservoir of S1P, during the coagulation of blood (40). The distribution of S1P and DHS1P contents (in decreasing order) of spinal cord > brain stem > cerebellum > cortex is quite intriguing. It has been reported that S1P receptors are expressed predominantly in brain white matter, rat cerebellum and cerebellar granule cells, and astrocytes (41–44). Therefore, the distribution of S1P and DHS1P closely parallels the expression levels of S1P receptors, indicating an interrelationship between sphingoid base-1-phosphate ligands and their receptors.

In summary, we have extended and applied shotgun lipidomics to the characterization and quantitation of S1P and DHS1P in crude lipid extracts in the presence of a low amount of ammonium hydroxide using precursor ion scanning of *m/z* 79.0 in the negative-ion mode. A broad linear dynamic range for the quantitation of both S1P and DHS1P and a detection limit at a low amol/μl concentration have been demonstrated using this approach. This method for the quantitation of sphingoid base-1-phosphates is generally simpler and more efficient than other previously published methods. The major advantages of this approach include the ability to *1*) avoid the use of radioisotopes, chromatographic separation, and chemical derivatization; *2*) obtain structural information of sphingoid base-1-phosphates; and *3*) detect S1P and DHS1P simultaneously. A potential limitation of this method is the possible interference of phosphate-containing lipid molecular species possessing an identical *m/z* to SIP and/or DHS1P. However, such interferences attributable to overlapping phosphate-containing lipid molecular species in mammalian samples are considered negligible. Accordingly, we anticipate that this methodology will be very useful for a wide range of physiological and pathophysiological studies.

TABLE 1. Mass contents of sphingoid base-1-phosphates in lipid extracts of mouse plasma and brain tissues

Sample	S1P	DHS1P
Plasma	1,310 ± 190	670 ± 40
Spinal cord	179 ± 10	101 ± 4
Brain stem	132 ± 14	68.7 ± 2.2
Cerebellum	80.5 ± 8.0	54.6 ± 6.7
Cortex	36.2 ± 2.1	49.9 ± 3.1

DHS1P, dihydrosphingosine-1-phosphate; S1P, sphingosine-1-phosphate. Total lipids of each biological sample were extracted using a modified Bligh and Dyer method in the presence of 5% acetic acid in the aqueous phase. Negative-ion tandem mass spectrometric analyses of sphingoid base-1-phosphates in the lipid extracts in the presence of 2% concentrated ammonium hydroxide were performed through scanning the first quadrupole in the mass range of interest and monitoring the third quadruple for the ion at *m/z* 79.0 while collisional activation was performed in the second quadrupole. Data represent means ± SEM of mouse plasma (pmol/ml) and brain tissues (pmol/mg protein) of four separate animals.

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REFERENCES

1. Pyne, S., and N. J. Pyne. 2000. Sphingosine 1-phosphate signalling in mammalian cells. *Biochem. J.* **349:** 385–402.
2. Le Stunff, H., S. Milstien, and S. Spiegel. 2004. Generation and metabolism of bioactive sphingosine-1-phosphate. *J. Cell. Biochem.* **92:** 882–899.
3. Rosen, H., and E. J. Goetzl. 2005. Sphingosine 1-phosphate and its receptors: an autocrine and paracrine network. *Nat. Rev. Immunol.* **5:** 560–570.
4. Watterson, K., H. Sankala, S. Milstien, and S. Spiegel. 2003. Pleiotropic actions of sphingosine-1-phosphate. *Prog. Lipid Res.* **42:** 344–357.
5. Colombaioni, L., and M. Garcia-Gil. 2004. Sphingolipid metabolites in neural signalling and function. *Brain Res. Brain Res. Rev.* **46:** 328–355.
6. Futerman, A. H., and Y. A. Hannun. 2004. The complex life of simple sphingolipids. *EMBO Rep.* **5:** 777–782.
7. Watterson, K. R., P. H. Ratz, and S. Spiegel. 2005. The role of sphingosine-1-phosphate in smooth muscle contraction. *Cell. Signal.* **17:** 289–298.
8. McVerry, B. J., and J. G. Garcia. 2004. Endothelial cell barrier regulation by sphingosine 1-phosphate. *J. Cell. Biochem.* **92:** 1075–1085.
9. Hla, T. 2004. Physiological and pathological actions of sphingosine 1-phosphate. *Semin. Cell Dev. Biol.* **15:** 513–520.
10. Goetzl, E. J., and H. Rosen. 2004. Regulation of immunity by lysosphingolipids and their G protein-coupled receptors. *J. Clin. Invest.* **114:** 1531–1537.
11. Spiegel, S., and S. Milstien. 2003. Exogenous and intracellularly generated sphingosine 1-phosphate can regulate cellular processes by divergent pathways. *Biochem. Soc. Trans.* **31:** 1216–1219.
12. Oskouian, B., and J. D. Saba. 2004. Death and taxis: what non-mammalian models tell us about sphingosine-1-phosphate. *Semin. Cell Dev. Biol.* **15:** 529–540.
13. Tamama, K., J. Kon, K. Sato, H. Tomura, A. Kuwabara, T. Kimura, T. Kanda, H. Ohta, M. Ui, I. Kobayashi, et al. 2001. Extracellular mechanism through the Edg family of receptors might be responsible for sphingosine-1-phosphate-induced regulation of DNA syn-

thesis and migration of rat aortic smooth-muscle cells. *Biochem. J.* **353:** 139–146.
14. Bu, S., M. Yamanaka, H. Pei, A. Bielawska, J. Bielawski, Y. A. Hannun, L. Obeid, and M. Trojanowska. 2006. Dihydrosphingosine 1-phosphate stimulates MMP1 gene expression via activation of ERK1/2-Ets1 pathway in human fibroblasts. *FASEB J.* **20:** 184–186.
15. Murata, N., K. Sato, J. Kon, H. Tomura, and F. Okajima. 2000. Quantitative measurement of sphingosine 1-phosphate by radioreceptor-binding assay. *Anal. Biochem.* **282:** 115–120.
16. Edsall, L. C., and S. Spiegel. 1999. Enzymatic measurement of sphingosine 1-phosphate. *Anal. Biochem.* **272:** 80–86.
17. Edsall, L., L. Vann, S. Milstien, and S. Spiegel. 2000. Enzymatic method for measurement of sphingosine 1-phosphate. *Methods Enzymol.* **312:** 9–16.
18. Yatomi, Y., F. Ruan, J. Ohta, R. J. Welch, S. Hakomori, and Y. Igarashi. 1995. Quantitative measurement of sphingosine 1-phosphate in biological samples by acylation with radioactive acetic anhydride. *Anal. Biochem.* **230:** 315–320.
19. Merrill, A. H., T. B. Caligan, E. Wang, K. Peters, and J. Ou. 2000. Analysis of sphingoid bases and sphingoid base 1-phosphates by high-performance liquid chromatography. *Methods Enzymol.* **312:** 3–9.
20. Caligan, T. B., K. Peters, J. Ou, E. Wang, J. Saba, and A. H. Merrill, Jr. 2000. A high-performance liquid chromatographic method to measure sphingosine 1-phosphate and related compounds from sphingosine kinase assays and other biological samples. *Anal. Biochem.* **281:** 36–44.
21. Ruwisch, L., M. Schafer-Korting, and B. Kleuser. 2001. An improved high-performance liquid chromatographic method for the determination of sphingosine-1-phosphate in complex biological materials. *Naunyn Schmiedebergs Arch. Pharmacol.* **363:** 358–363.
22. Min, J. K., H. S. Yoo, E. Y. Lee, W. J. Lee, and Y. M. Lee. 2002. Simultaneous quantitative analysis of sphingoid base 1-phosphates in biological samples by o-phthalaldehyde precolumn derivatization after dephosphorylation with alkaline phosphatase. *Anal. Biochem.* **303:** 167–175.
23. He, X., A. Dagan, S. Gatt, and E. H. Schuchman. 2005. Simultaneous quantitative analysis of ceramide and sphingosine in mouse blood by naphthalene-2,3-dicarboxyaldehyde derivatization after hydrolysis with ceramidase. *Anal. Biochem.* **340:** 113–122.
24. Butter, J. J., R. P. Koopmans, and M. C. Michel. 2005. A rapid and validated HPLC method to quantify sphingosine 1-phosphate in human plasma using solid-phase extraction followed by derivatization with fluorescence detection. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* **824:** 65–70.
25. Van Veldhoven, P. P., P. De Ceuster, R. Rozenberg, G. P. Mannaerts, and E. de Hoffmann. 1994. On the presence of phosphorylated sphingoid bases in rat tissues. A mass-spectrometric approach. *FEBS Lett.* **350:** 91–95.
26. Mano, N., Y. Oda, K. Yamada, N. Asakawa, and K. Katayama. 1997. Simultaneous quantitative determination method for sphingolipid metabolites by liquid chromatography/ionspray ionization tandem mass spectrometry. *Anal. Biochem.* **244:** 291–300.
27. Sullards, M. C., and A. H. Merrill, Jr. 2001. Analysis of sphingosine 1-phosphate, ceramides, and other bioactive sphingolipids by high-performance liquid chromatography-tandem mass spectrometry. *Sci. STKE.* **2001:** PL1.
28. Sullards, M. C., E. Wang, Q. Peng, and A. H. Merrill, Jr. 2003. Metabolomic profiling of sphingolipids in human glioma cell lines by liquid chromatography tandem mass spectrometry. *Cell. Mol. Biol.* **49:** 789–797.
29. Pettus, B. J., J. Bielawski, A. M. Porcelli, D. L. Reames, K. R. Johnson, J. Morrow, C. E. Chalfant, L. M. Obeid, and Y. A. Hannun. 2003. The sphingosine kinase 1/sphingosine-1-phosphate pathway mediates COX-2 induction and PGE2 production in response to TNF-alpha. *FASEB J.* **17:** 1411–1421.
30. Esch, S. W., T. D. Williams, S. Biswas, A. Chakrabarty, and S. M. Levine. 2003. Sphingolipid profile in the CNS of the twitcher (globoid cell leukodystrophy) mouse: a lipidomics approach. *Cell. Mol. Biol.* **49:** 779–787.
31. Berdyshev, E. V., I. A. Gorshkova, J. G. Garcia, V. Natarajan, and W. C. Hubbard. 2005. Quantitative analysis of sphingoid base-1-phosphates as bisacetylated derivatives by liquid chromatography-tandem mass spectrometry. *Anal. Biochem.* **339:** 129–136.
32. Han, X., and R. W. Gross. 2003. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics. *J. Lipid Res.* **44:** 1071–1079.
33. Han, X., J. Yang, H. Cheng, H. Ye, and R. W. Gross. 2004. Towards fingerprinting cellular lipidomes directly from biological samples by two-dimensional electrospray ionization mass spectrometry. *Anal. Biochem.* **330:** 317–331.
34. Han, X., and R. W. Gross. 2005. Shotgun lipidomics: electrospray ionization mass spectrometric analysis and quantitation of the cellular lipidomes directly from crude extracts of biological samples. *Mass Spectrom. Rev.* **24:** 367–412.
35. Han, X., and R. W. Gross. 2005. Shotgun lipidomics: multi-dimensional mass spectrometric analysis of cellular lipidomes. *Expert Rev. Proteomics.* **2:** 253–264.
36. Han, X., K. Yang, J. Yang, K. N. Fikes, H. Cheng, and R. W. Gross. 2006. Factors influencing the electrospray intrasource separation and selective ionization of glycerophospholipids. *J. Am. Soc. Mass Spectrom.* **17:** 264–274.
37. Cheng, H., S. Guan, and X. Han. 2006. Abundance of triacylglycerols in ganglia and their depletion in diabetic mice: implications for the role of altered triacylglycerols in diabetic neuropathy. *J. Neurochem.* **97:** 1288–1300.
38. Sullards, M. C. 2000. Analysis of sphingomyelin, glucosylceramide, ceramide, sphingosine, and sphingosine 1-phosphate by tandem mass spectrometry. *Methods Enzymol.* **312:** 32–45.
39. Han, X. 2002. Characterization and direct quantitation of ceramide molecular species from lipid extracts of biological samples by electrospray ionization tandem mass spectrometry. *Anal. Biochem.* **302:** 199–212.
40. Kobayashi, N., T. Nishi, T. Hirata, A. Kihara, T. Sano, Y. Igarashi, and A. Yamaguchi. 2006. Sphingosine 1-phosphate is released from the cytosol of rat platelets in a carrier-mediated manner. *J. Lipid Res.* **47:** 614–621.
41. Liu, C. H., and T. Hla. 1997. The mouse gene for the inducible G-protein-coupled receptor edg-1. *Genomics.* **43:** 15–24.
42. Zhang, G., J. J. Contos, J. A. Weiner, N. Fukushima, and J. Chun. 1999. Comparative analysis of three murine G-protein coupled receptors activated by sphingosine-1-phosphate. *Gene.* **227:** 89–99.
43. Anelli, V., R. Bassi, G. Tettamanti, P. Viani, and L. Riboni. 2005. Extracellular release of newly synthesized sphingosine-1-phosphate by cerebellar granule cells and astrocytes. *J. Neurochem.* **92:** 1204–1215.
44. Chae, S. S., R. L. Proia, and T. Hla. 2004. Constitutive expression of the S1P1 receptor in adult tissues. *Prostaglandins Other Lipid Mediat.* **73:** 141–150.