A procedure for solid phase extractions using metal oxide coated silica column in lipidomics

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ABSTRACT: Lipid enrichment is often indispensable to enhance the coverage of targeted molecules in mass spectrometry (MS)-based lipidomics studies. This study developed a simple stepwise fractionation method using titanium and zirconium dioxide coated solid phase (SP) silica column that separates neutral lipids, phospholipids, and other lipids including glycolipids and several polar lipids such as sulfatides. Our procedure used chloroform to dissolve lipids and the neutral lipids including cholesteryl ester, di- and triacyl glycerols were collected in the loading fraction. Secondly, methanol with formic acid (99:1, v/v) retrieved sphingolipids, glycolipids, and fatty acids by competing for affinity with Lewis acid sites on the metal oxide surface. Finally, phospholipids strongly retained via chemoaffinity interaction were eluted using a solution containing 5% ammonia and high water content (45:50 v/v, 2-propanol:water), which cancels electrostatic- and chelating interactions with the SP column. The high average reproducibility of <10% and the coverage of ~100% compared to non-SP extracts were demonstrated by untargeted lipidomics of human plasma, and mouse brain and feces. The advantage of our procedure was showcased by characterizing minor lipid subclasses mono- and digalactosyl monoacylglycerols in feces and acetylated- and glycolylated derivatives of gangliosides in brain that were not detected in conventional monophase or liquid-liquid extraction methods. Likewise, the value of our method in biology is maximized during glycolipidome profiling by the absence of phospholipids and glycolipids that cover >80% of the chromatographic peaks.

Lipids serve as membrane components, signal transduction and energy storage materials in living organisms. Liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS)-based untargeted lipidomics has made remarkable progress in understanding the diversity of lipids, with ~50,000 lipid molecules currently registered in LIPID MAPS [1]. However, most lipids have low abundant MS signals, whose low-quality MS/MS spectra increase false hits in the annotation process. This is owing to the wide range of lipid concentrations, such as $>10^8$ orders of lipid dynamic range in human plasma [2]. In fact, a small fraction of highly abundant lipid molecules (glycerolipids and phospholipids) obscure the ions of the other diverse lipids. This makes it difficult to discover new metabolites that were previously unknown, since high quality MS/MS spectra is preferred to elucidate lipid structures.

This issue may be overcome by improving the fractionation, enrichment, and chromatographic separation of the lipid extract to reduce matrix effects such as ion suppression that improve the sensitivity of minor lipids. Preparative LC and thin-layer chromatography are popular techniques to enrich the metabolite of interest [3,4], although they are time-consuming processes that need to be optimized for the targeted structure. Alkaline hydrolysis of glycerolipids was used for the highly sensitive analysis of glycosyl sphingolipids to exploit the difference in bond stability between the amide bond (*N*-acyl chain in sphingolipids) and the ester bond (*O*-acyl chain in glycerolipids and phospholipids) [5]. However, the technique is limited to glycolipid analysis where the major glycerolipids become out of the scope in lipid profiling.

Solid phase extraction (SPE) is a popular technique in sample preparation although the application is rarely reported in lipidomics. Titanium dioxide (TiO_2) is used to selectively enrich compounds that contain a phosphoryl group whose lone pair (Lewis base) has the interaction with the empty *d* orbital of the TiO_2 transition metal (Lewis acid) [6]. TiO_2 was widely used to enrich phosphopeptides [7] and phospholipids [8,9]. Recently, TiO_2 coated silica column is a potential tool to fractionate glycolipids [10,11]. Glycolipids exhibit high affinity with a metal oxide column via the hydroxyl group of sugar component under neutral conditions, whereas phospholipid affinity increases in acidic conditions. Therefore, optimizing loading- and elution solvents in the use of TiO_2 column would facilitate a simple- and effective fractionation of lipids based on the polar head categories.

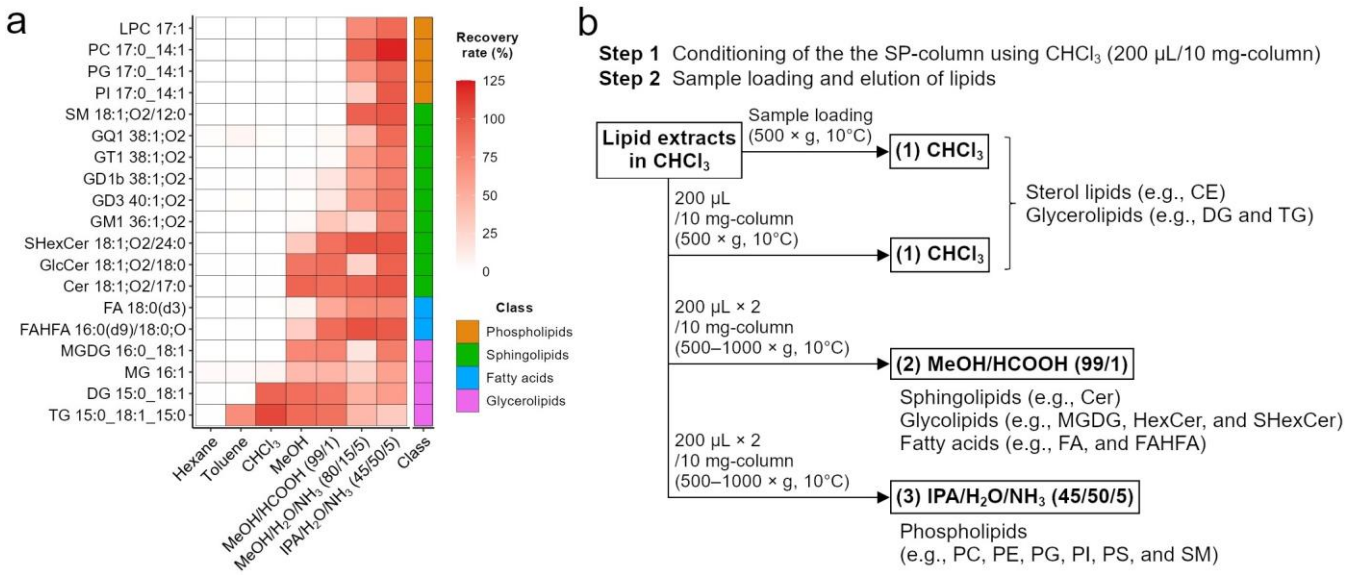

Figure 1. Development of the stepwise fractionation. (a) Recovery rate of the loading fraction obtained by dissolving the lipid standards in each solvent. (b) Workflow of the lipid fractionation.

This study developed a procedure to separate lipids into three groups by using a monolithic silica column coated with TiO_2 and zirconium dioxide (ZrO_2). Zr has a strong chemical affinity with phosphate group [12]; therefore, glycolipids and phospholipids fractionations are facilitated [13]. The silica phase of the column represents electrostatic interactions with the polar moiety of lipids that help to enrich glycolipids. We particularly examined the efficiency of our SPE technique for glycerolipids [monoacylglycerols (MGs), diacylglycerols (DGs) and triacylglycerols (TGs)], sphingolipids [ceramides (Cers), hexosylceramides (HexCers), and some gangliosides], and phospholipids [phosphatidylcholines (PCs), phosphatidylethanolamine (PEs), phosphatidylglycerols (PGs), phosphatidylserines (PSs), phosphatidylinositols (PIs) and sphingomyelins (SMs)] by using the authentic standards. The scalability was evaluated using the biological samples including human plasma, mouse brain, and feces.

EXPERIMENTAL SECTION

Chemicals and Reagents. Reagent grade ammonia solution (NH_3) and chloroform ($CHCl_3$), ammonium acetate solution for high performance LC (HPLC), formic acid (HCOOH) for LC/MS, acetonitrile (MeCN), methanol (MeOH), 2-propanol (IPA), and ultrapure water (H_2O) for quadrupole time-of-flight MS (QTOF-MS) were obtained from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). Toluene for HPLC was obtained from Sigma-Aldrich Japan (Tokyo, Japan). MonoSpin Phospholipid was obtained from GL Sciences Inc. (Tokyo, Japan) for the monolithic silica column coated TiO_2 and ZrO_2. Individual lipid synthetic standards and EquiSPLASH containing equal concentrations of deuterium-labeled lipid standard (100 µg/mL) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL, U.S.A.). Human plasma (pool, heparin sodium) was obtained from Cosmo Bio Co., LTD. (Tokyo, Japan).

Animals. All animal experiments were performed in accordance with the ethical protocol approved by the Tokyo University of Agriculture and Technology (R5-50). C57BL/6J male mice were purchased from SLC (Shizuoka, Japan). Mice were fed the chow of CE-2 (CLEA Japan, Tokyo, Japan). The brain and feces were harvested and immediately frozen after dissection and stored at −80°C until lipid extraction.

Monophasic liquid extraction. A monophase lipid extraction method was performed according to a previous method [14]. Samples were mixed with 200 µL of MeOH and extracted using ultrasonication for 5 min. One hundred microliters of $CHCl_3$ was added and the solution was vortexed for 1 min and extracted on ice for 60 min. The solution was further mixed with 20 µL of H_2O and vortexed for 1 min. The supernatant was collected after centrifugation at 16,000 g for 5 min at 4°C. Lipid extracts were dried using a centrifugal evaporator.

Biphasic liquid extraction. The Bligh and Dyer method was performed according to a previous method with minor modifications [15]. Samples were mixed with 1,000 µL of MeOH/$CHCl_3$/H_2O (10:4:4, v/v/v). Lipids were extracted using a vortex mixer for 1 min and ultrasonicated for 5 min. The solution was centrifuged at 16,000 g for 5 min at 4°C, and 700 µL of the supernatant was transferred to a clean tube. The supernatant was mixed with 235 µL of $CHCl_3$ and 155 µL of H_2O using a vortex mixer for 1 min. Both layers were collected after centrifugation at 16,000 g for 5 min at 4°C. Lipid extracts were dried with a centrifugal evaporator.

Solid phase enrichment. A dried lipid extract was dissolved by applying 100 µL of loading solvent and 50 µL of the sample was loaded to the SP column. An equal amount of EquiSPLASH was added to each sample before purifying the lipid extract. The detailed protocol developed in the present study was described in the Results and Discussion. All fractions collected via SPE were dried with a centrifugal evaporator and dissolved in MeOH containing Cer 18:1;O2/17:0 standard for LC-MS analysis.

Lipidome analysis. The LC/QTOF-MS system was composed of an Exion LC and a ZenoTOF 7600 with an electrospray ionization ion source (SCIEX, Framingham, MA, U.S.A.). The LC conditions were as follows: injection volume, 1 µL; mobile phase, MeCN/MeOH/H_2O (1:1:3, v/v/v) (A) and MeCN/IPA (1:9, v/v) (B) (both contained 10 nM of ethylenediaminetetraacetic acid and 5 mM of ammonium acetate); flow rate, 300 µL/min; column; Unison UK-C18 MF (50 × 2.0

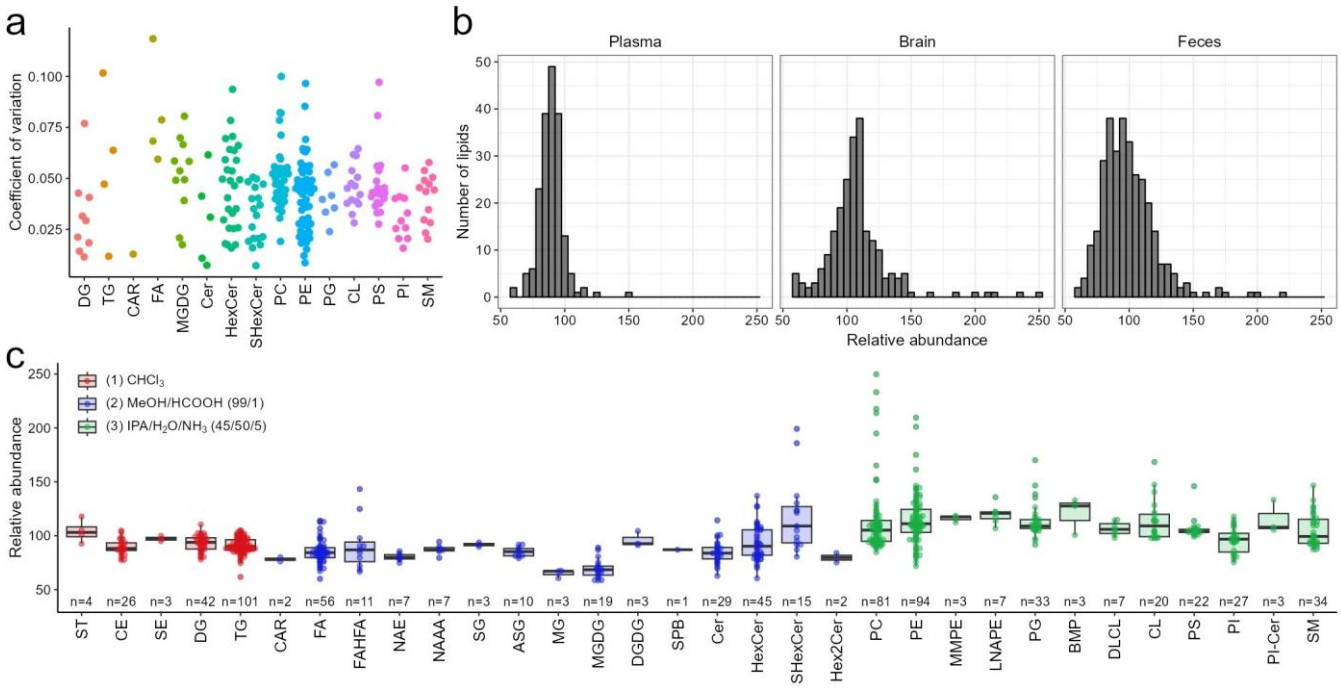

Figure 2. Lipidome analysis of biological samples. (a) Evaluation of repeatability using the mouse brain extract. The coefficient of variation was evaluated using the SPE replicates (n = 4). In addition to the internal standard subclasses, acyl-carnitine (CAR), bis(monoacylglycero)phosphate (BMP), and cardiolipin (CL) were evaluated. (b) Distribution of relative abundance in each sample. Relative abundance was evaluated by comparison with lipidomics data of non-SP extracts. (c) Lipidome analysis of each fraction. Lipidomics data from three biological samples were combined by lipid subclass. The relative abundance was shown in the representative fractions. Sterol (ST), steryl ester (SE), *N*-acyl-ethanolamines (NAE), *N*-acyl amino acid (NAAA), sterylglucoside (SG), acyl-SG (ASG), sphingoid base (SPB), monomethyl-PE (MMPE), *N*-acyl-lyso-PE (LNAPE) and dilyso-CL (DLCL) were also collected by SPE.

mm, 3 µm, Imtakt Corp., Kyoto, Japan); gradient, 0.5% (B) (1 min), 0.5–40% (B) (4 min), 40–64% (B) (2.5 min), 64–71% (B) (4.5 min), 71–82.5% (B) (0.5 min), 82.5–85% (B) (6.5 min), 85–99% (B) (1 min), 99% (B) (2 min), 99–0.5% (B) (0.1 min), 0.5% (B) (2.9 min); column oven temperature, 45°C. Mass spectrometry conditions were as follows: ion source gas 1, 50 psi; ion source gas 2, 50 psi; curtain gas, 35 psi; CAD gas, 7; source temperature, 300°C; spray voltage, −4500 V (negative); *m/z* range of TOF-MS, 75–1250 Da (positive) or 100–1700 Da (negative); declustering potential, −80 V; collision energy, 42 ± 15 eV; Q1 resolution, unit; acquisition mode, data dependent MS/MS; accumulation time, 50 msec.

Data analysis. Lipid molecules were annotated using MS-DIAL version 5.1 [14]. Representative parameters were as follows: minimum peak height for peak picking, 100; retention time (RT) tolerance for peak alignment, 0.15 min; MS1 tolerance, 0.01 Da; MS2 tolerance, 0.025 Da (Table S1). The adduct ions used in each lipid subclass are listed in Table S2. The annotated results were manually curated. The data matrix was prepared by the mean value of analytical replicates (n = 4) after normalizing the lipid molecules based on the sample volume and ion abundance of Cer 18:1;O2/17:0 standard (Table S3). The lipidome results are summarized in Tables S4−7.

RESULTS AND DISCUSSION

Optimization of the fractionation protocol. Lewis acidic sites on the metal oxide surface have affinity with lone pair heteroatoms. Seven solutions including hexane, toluene, $CHCl_3$, MeOH, MeOH/HCOOH (99/1, v/v), MeOH/H_2O/NH_3 (80/15/5, v/v/v) and IPA/H_2O/NH_3 (45/50/5, v/v/v) were investigated to determine the lipid solubility and interaction with stationary phases by using mouse brain extract and synthetic standards. Hexane, toluene, and $CHCl_3$ are commonly used in LC-based lipidomics, and exhibited sufficient solubility for a wide range of lipid subclasses (Figure S1). Although MeCN was a suitable solvent for the adsorption of lipids (except for glycerolipids) on the TiO_2 column in a previous report [10], the solubility of lipids was insufficient when MeCN was used in our study (except for Cer, DG, fatty acid (FA), PC, PG, and TG). The optimal solvent for loading the lipid extract was determined based on the solubility. Lipid molecules are mainly bound to the stationary phase in a silica-based column by electrostatic interactions. The elution behavior of cholesteryl esters (CEs), TGs, DGs, Cers, and glucosylceramides (GlcCers) depend on the polarity of the solvent among four solvents (hexane, toluene, $CHCl_3$, and MeOH) (Figure 1a). We selected $CHCl_3$ as the suitable solvent to dissolve lipids and to elute the major neutral lipids of DGs, TGs, and CEs while retaining sphingolipids and phospholipids.

Secondly, the fractionation of lipid groups with hydroxyl and/or carboxyl groups such as FAs, sphingolipids, and glycolipids was investigated. The phosphate group has the strongest chemical affinity for the metal oxide out of the three functional groups (phosphate, hydroxyl, and carboxyl groups). The addition of HCOOH affects the competition with organic ligands since HCOOH has a carboxyl group that has a chemical affinity with metal oxide. MeOH with HCOOH effectively eluted FAs, FA esters of hydroxy FAs (FAHFAs), sulfatides (SHexCers), and some gangliosides (such as the GM and GD series) with high recovery (Figure 1a and Figure S2) compared

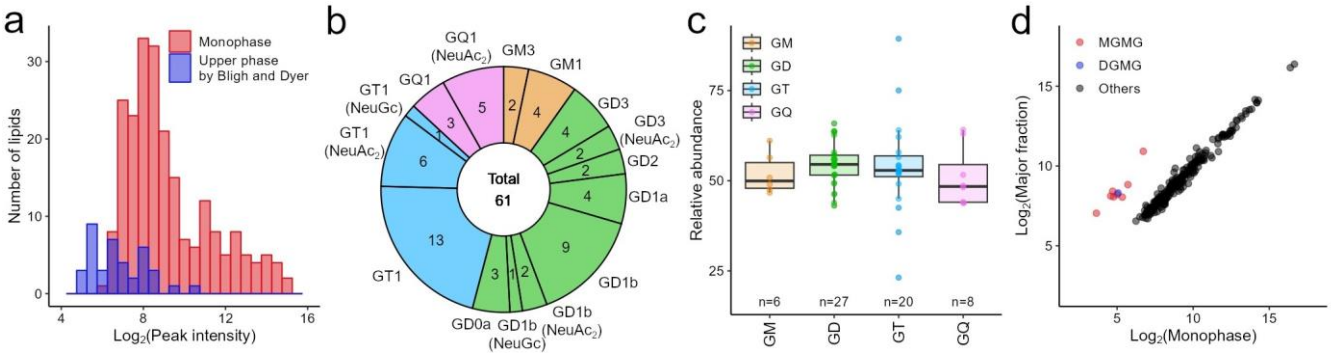

Figure 3. Glycolipid analysis using the fractionation method. (a) Distribution of lipids in the mouse brain extract. (b) Number of gangliosides detected in the mouse brain. Three sialic acids [*N*-acetylneuraminic acid (NeuAc), $NeuAc_2$ and *N*-glycolylneuraminic acid (NeuGc)] were detected in this study. (c) Ganglioside profiling of the mouse brain. Relative abundance was evaluated by comparing with lipidomics data of non-SP extracts. (d) Comparison of lipidomics data between monophase- and SP extractions obtained from the mouse feces.

to MeOH without HCOOH. A previous study describes that acidic solvents have a strong affinity with phospholipids and weak interaction with glycolipids using a TiO_2 column [11]. Therefore, MeOH/HCOOH (99/1, v/v) was found to be the optimal solvent to elute FA metabolites, sphingolipids, and glycolipids. HCOOH can be easily removed in the drying process using a centrifugal evaporator. Therefore, the throughput and cost for purification were reduced by avoiding the removal step of counter substances (such as 2,5-dihydroxybenzoic acid [13]) using other SP columns.

The phosphate group has strong Lewis basic sites. Therefore, phospholipid elution can be achieved using a solvent containing NH_3 that removes the chelating interaction. Most phospholipids were eluted using MeOH/H_2O/NH_3 (80/15/5, v/v/v), while PIs with six hydroxyl groups in the inositol moiety were retained by the strong affinity with metal oxide columns. Electrostatic and chelating interactions need to be canceled by hydration of polar groups and an ammonium ion, respectively, to elute lipids having rich polar functional groups. Therefore, a solvent containing a high amount of H_2O was used while retaining lipid solubility by changing the solvent from MeOH to IPA (Figure S1a). Finally, IPA/H_2O/NH_3 (45/50/5 v/v/v) was determined as the optimal solution for a high recovery rate of these lipids (Figure 1a).

We proposed a procedure for stepwise purification of lipid subclasses (Figure 1b). The reproducibility of the SPE procedure was within 10% in the lower phase of mouse brain extract using the Bligh and Dyer method (Figure 2a). The recovery rate of constituent lipid molecules was normalized by the internal standards of corresponding lipid subclasses (where available) whose internal standards were added in the lipid extraction process (Figure S3). Meanwhile, mixed solvents with NH_3 caused acyl migration of lyso-phospholipids and alkaline hydrolysis of phospholipids (Figure S1). Therefore, major lyso-phospholipids were excluded from further experiments in this study.

Application to untargeted lipidomics. The optimized SPE procedure was applied to human plasma, mouse brain- and feces to evaluate the performance of SPE with respect to a wide variety of lipid subclasses (Figure 2). The sample volume of each extraction protocol injected on the column was kept constant to evaluate the degree of ionization suppression and the recovery rate of each lipid extract. There was no significant difference between monophasic and biphasic extractions using the current reversed phase LC/MS system (Figure S4a). Therefore, the differences of plasma, brain and feces samples were investigated using monophasic extraction. Their distributions were mostly similar among three samples when evaluating the relative abundance between before and after purification of lipids using the SPE method (Figure 2b). Lipids were fractionated with high recovery into three optimized solvents, despite the difference of sample matrix (Figure 2c). This was consistent with the synthetic standards.

Various gangliosides are reported in biological samples [16,17], though most gangliosides are rarely detected in a conventional monophasic extraction owing to the dynamic range of MS (Figure 3a and Table S5). Gangliosides were eluted into either MeOH/HCOOH (99/1, v/v) or IPA/H_2O/NH_3 (45/50/5, v/v/v) fractions based on the number of sialic acids and sugar motifs containing the hydroxyl and carboxyl groups as the Lewis basic sites (Figure 1a). Therefore, a wide range of gangliosides could be simultaneously fractionated by collecting IPA/H_2O/NH_3 (45/50/5, v/v/v) immediately after loading the upper phase of Bligh and Dyer method (Figure 3b, 3c, and Table S7). By using the procedure, the acetylated- and glycolylated derivatives of GD1a, GD1b, GT1, and GQ1 gangliosides described as $NeuAC_2$ and NeuGc, respectively, were newly characterized, while they were not in the scope of the MS-DIAL lipidomics pipeline. Moreover, feces analysis detected monogalactosyl DG (MGDG) and digalactosyl DG (DGDG) before and after purification of the sample, whereas monogalactosyl MG (MGMG) and digalactosyl MG (DGMG) were only detected in the MeOH/HCOOH (99/1, v/v) fraction of mouse feces (Figure 3d). Likewise, the stepwise fractionation should be an important tool for the exploratory study of glycolipidome since the removal of salts and polar metabolites using solvent extraction and phospholipids using SPE decrease ion suppression in MS analysis (Figure S4b−4e) [18,19].

CONCLUSION

We developed a simple and effective lipid separation method using TiO_2 and ZrO_2 coated silica column, wherein four lipid categories (gangliosides, neutral lipids, phospholipids, and other lipids including glycolipids) are fractionated, while SPE is often used to divide a mixture into only two categories. In addition, the recovery rate of lipid molecules in plasma, brain and feces samples was within 80–120%. This stepwise solid phase fractionation contributes to reducing false positive

discovery in untargeted lipidomics by combining the knowledge of separation behavior based on the functional groups. In addition, the SPE method in the micro scale can accelerate structural analysis of novel compounds by using advanced fragmentation techniques such as electron activated dissociation [20]. There are few reports involving high recovery glycolipid fractionation. Therefore, this technique may facilitate the discovery of novel glycolipid molecules in biological samples, such as characterizing ganglioside derivatives, MGMG, and DGMG obtained in this study. The untargeted lipidomics of an enriched fraction, named untargeted "probing" lipidomics facilitates the discovery of novel lipid metabolites associated with phenotypes. Furthermore, lipidomics using low-flow LC (<10 μL/min), wherein a simplified sample matrix is preferable for a sustainable operation, can be accelerated by using our fractionation procedure.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at ACS Publications website.

Data availability

All raw MS data are available on the RIKEN DROPMet website (http://prime.psc.riken.jp/menta.cgi/prime/drop_index) under the index number DM0051. The source code of MS-DIAL is available at https://github.com/systemsomicslab/MsdialWorkbench.

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Author Contributions

Hiroaki Takeda and Hiroshi Tsugawa designed the research. M.H. and J.M. prepared the animals. Hiroaki Takeda and M.T. extracted samples, analyzed lipids, and performed data analysis. Hiroaki Takeda and Hiroshi Tsugawa wrote the manuscript and prepared the figures. All authors have given approval to the final version of the manuscript.

ACKNOWLEDGMENT

The authors thank Dr. Masahiro Furuno (Osaka University) and Dr. Shigenori Ota (GL Sciences Inc.) for technical advice about chemoaffinity. This research was supported by Japan Agency for Medical Research and Development (AMED) under the Brain Mapping by Integrated Neurotechnologies for Disease Studies (Brain/MINDS) (JP15dm0207001) and Japan Science and Technology Agency (JST) Exploratory Research for Advanced Technology (ERATO) (JPMJER2101).

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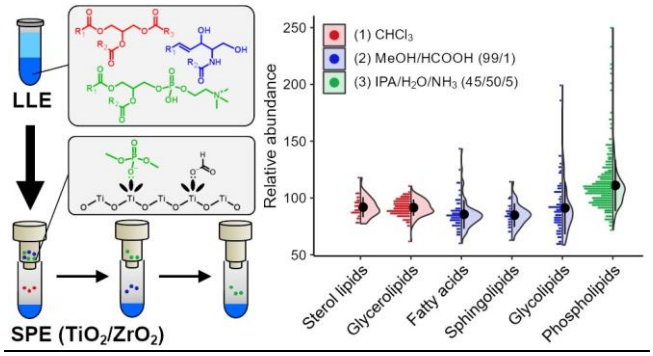
Table of Contents artwork