Chemistry and Physics of Lipids 223 (2019) 104787

Contents lists available at ScienceDirect

Chemistry and Physics of Lipids

journal homepage: www.elsevier.com/locate/chemphyslip

Comparative evaluation of the extraction and analysis of urinary phospholipids and lysophospholipids using MALDI-TOF/MS

Xin Li[a], Kenji Nakayama[a,b,*], Takayuki Goto[b], Shusuke Akamatsu[a], Koji Shimizu[c], Osamu Ogawa[a], Takahiro Inoue[a]

[a] Department of Urology, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan
[b] Support Center for Precision Medicine, Shimadzu Techno-Research, Inc., 1 Nishinokyou-Shimoai-cho, Nakagyou-ku, Kyoto 604-8436, Japan
[c] Clinical Research Center for Medical Equipment Development, Kyoto University Hospital, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan

ARTICLE INFO

Keywords:
Lipid
Urine
MALDI-TOF/MS
Comprehensive screening
Quantitative analysis
Diagnosis

ABSTRACT

Lipids, particularly phospholipids (PLs) and lysophospholipids (LPLs), are attracting increasing scientific interest for their biological functions in cells and their potential as disease biomarkers for Alzheimer's disease and several types of cancer. Urinary PLs and LPLs could be ideal clinical biomarkers, because urine can be collected easily and noninvasively. However, due to their very low concentrations in urine compared with the relatively large quantity of contaminants in this matrix, efficient extraction and sensitive detection are required for analyzing urinary PLs and LPLs. In this study, various methods for analyzing PLs and LPLs in urine were compared and optimized from a clinical perspective.

An optimized lipid extraction method and a matrix for matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) were established using two external ionization standards and an internal standard mix containing 13 human urinary lipids. 9-Aminoacridine (9-AA) was a useful and effective matrix for the MALDI-TOF/MS analysis of all the internal standard lipids in both positive and negative ion modes. However, it was necessary to determine the proportional lipid concentrations from the balance between the extracted lipid and the matrix. The extraction efficiency and reproducibility of the acidified Bligh and Dyer method were excellent for both positively and negatively charged lipids. Analysis of small volumes of urine was the most efficient with the 9-AA MALDI matrix at concentrations of or below 5 mM. The combined analytical procedures allowed rapid and comprehensive screening of low concentrations of PLs and LPLs in clinical samples.

1. Introduction

Lipids, particularly phospholipids (PLs) and lysophospholipids (LPLs), are attracting increasing interest because of their vital physiological functions such as signal transduction (Zhou et al., 2015), vesicular trafficking (Campa and Hirsch, 2017), apoptosis (Nagata et al., 2016), and energy storage (Vanni, 2017) and their structural functions such as maintaining cellular and organellar membrane integrity (Edidin, 1997; Vanni, 2017). PLs and LPLs are recognized for their potential as biomarkers and for their involvement in the progression of various diseases, such as colorectal cancer (Zhao et al., 2007), prostate cancer (Min et al., 2011), breast cancer (Kim et al., 2009), Alzheimer's disease (Mulder et al., 2003; Orešič et al., 2011), cardiovascular disease (Stübiger et al., 2012), obesity, and diabetes (Barber et al., 2012). Particularly, urinary PLs and LPLs could be useful as clinical biomarkers, because urine can be collected easily and noninvasively (Kim et al., 2008). However, due to the high structural diversity of these molecules and their very low concentrations in urine, development of efficient extraction and sensitive analytical methods is highly desirable.

Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/MS) has been used for lipid analysis since it was introduced in the late 1980s (Solouki et al., 1995). This technique

Abbreviations: MALDI-TOF/MS, matrix assisted laser desorption/ionization time-of-flight mass spectrometry; LC/MS, liquid chromatography mass spectrometry; HPLC, high performance liquid chromatography; MeOH, methanol; MTBE, methyl-tert-butyl ether; ACN, acetonitrile; TFA, trifluoroacetic acid; 9-AA, 9-aminoacridine; DHB, 2,5-dihydroxybenzoic acid; STD, standard; LDL, low-density lipoproteins; PL, phospholipid; LPL, lysophospholipid; LPC, lysophosphatidylcholine; LPA, lysophosphatidic acid; LPS, lysophosphatidylserine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; SM, sphingomyelin; PC, phosphatidylcholine; PA, phosphatidic acid; PS, phosphatidylserine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol

* Corresponding author at: Department of Urology, Graduate School of Medicine, Kyoto University, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan.
E-mail address: knakayam@kuhp.kyoto-u.ac.jp (K. Nakayama).

https://doi.org/10.1016/j.chemphyslip.2019.104787
Received 20 February 2019; Received in revised form 19 May 2019; Accepted 22 May 2019
Available online 27 June 2019
0009-3084/

is operationally simple and advantageous for lipid analysis because it allows soft ionization, short analysis times, and excellent sensitivity. In addition, samples can be reanalyzed even at a later time (Schiller et al., 2004, 2007; Fuchs et al., 2010; Leopold et al., 2018). However, lipid analysis using this technique also has several disadvantages that makes quantitative measurements quite difficult (Fuchs et al., 2010; Szájli et al., 2008). These include a strong matrix background in the low *m/z* region, formation of numerous lipid adducts, and poor reproducibility. Significant efforts have been undertaken recently to overcome these drawbacks, such as developing novel matrices and better spotting techniques. A neutral matrix, 9-aminoacridine (9-AA), has been introduced for the selective analysis of different classes of lipids under positive and negative ionization modes. 9-AA can afford better signal-to-noise ratio and lower matrix background in the low *m/z* range than some of the commonly used matrices, such as α-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) (Sun et al., 2008; Bresler et al., 2011). The use of 9-AA as a matrix has enabled highly selective ionization of some of the classes of lipids, including phosphatidylcholine (PC), lysophosphatidylcholine (LPC), and sphingomyelin (SM), in the positive ion mode. Highly sensitive detection for the analysis of negatively charged lipids, including phosphatidylinositol (PI) and sulfatide (ST), has also been demonstrated with 9-AA (Sun et al., 2008; Pyttel et al., 2012; Leopold et al., 2018). Moreover, analysis with 9-AA in isopropanol:acetonitrile (60:40, v/v) has yielded accurate quantitative results for PLs and triglyceride (TG) standard species, without significant background interference (Sun et al., 2008). In several recent studies, the sensitivity of detection in the negative mode was even higher for the anionic phospholipids, phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI), when 9-AA was used as a matrix (Angelini et al., 2012; Serna et al., 2015).

Extraction of PLs and LPLs from a biological matrix using an appropriate organic solvent system is a critical step for the successful analysis of these compounds. Preventing the degradation of lipid target and introduction of inorganic contaminants is crucial for an effective extraction. Lipid extraction has traditionally been carried out with biofluids or tissues using a mixture of chloroform and methanol. This type of extraction was introduced over 50 years ago by Folch (Folch et al., 1957) and Bligh and Dyer (Bligh and Dyer, 1959). These protocols have been modified thereafter using less hazardous solvents, such as hexane-isopropanol mixture (Hara and Radin, 1978), methyl-tert-butyl ether (Matyash et al., 2008), and mixture of butanol and methanol (Löfgren et al., 2012), to improve the extraction efficiency.

For the comprehensive analysis of different classes of lipids in biological samples, it is necessary to compare the analytical procedures in terms of recovery, reproducibility, and artifacts. Extraction methods for isolation of lipids from plasma (Byeon et al., 2012), cells (Bi et al., 2013), and low-density lipoproteins (LDL) (Reis et al., 2013) have been examined in multiple studies over the years. Due to the very low concentrations of lipids compared with the relatively high concentrations of contaminants in urine samples, the analysis of PLs and LPLs in small volumes (≤1 mL) of urine samples has been quite challenging. Therefore, we compared methods for the analysis of urinary PLs and LPLs and optimized them from a clinical perspective for applications in molecular diagnosis.

2. Materials and methods

2.1. Materials

Ultrapure water (Fisher Optima grade, Fisher Scientific, Pittsburgh, PA) was used for liquid chromatography (LC)-MS. HPLC-grade chloroform ($CHCl_3$), methanol (MeOH), methyl-tert-butyl ether (MTBE), butanol, heptane, ethyl acetate, acetonitrile (ACN), hexane, isopropanol (IPA), and trifluoroacetic acid (TFA) were purchased from Fujifilm Wako Pure Chemical Corp. (Osaka, Japan). Proteomics-grade hydrochloric acid (HCl) was also obtained from Fujifilm Wako Pure Chemical Corp. 9-Aminoacridine (9-AA) and 8 M molecular biology-grade lithium chloride (LiCl) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2,5-Dihydroxybenzoic acid (DHB) was obtained from LaserBio Labs (Sophia Antipolis Cedex, France). All lipid internal standards were purchased from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and are listed in Table 1-1. The internal standards were representatives of the following lipid classes: lysophosphatidylcholine [LPC (17:1)]; sphingomyelin [SM (d18:1/17:0)]; phosphatidylcholine [PC (24:0/24:0)]; lysophosphatidic acid [LPA (17:1)]; lysophosphatidylserine [LPS (17:1)]; lysophosphatidylethanolamine [LPE (17:1)]; lysophosphatidylglycerol [LPG (17:1)]; lysophosphatidylinositol [LPI (17:1)]; phosphatidic acid [PA (16:0/18:1)]; phosphatidylserine [PS (17:0/14:1)]; phosphatidylethanolamine [PE (17:0/20:4)]; phosphatidylglycerol [PG (17:0/20:4)]; and phosphatidylinositol [PI (21:0/22:6)]. PC (14:0/14:0) and PG (17:0/14:1) were obtained from Avanti Polar Lipids, Inc. and used as external ionization standards (Table 1-2).

Table 1
Lipid standard mixtures.

1-1. Internal lipid standards (internal STDs)

Standards	Concentration (μM)	Detected *m/z*
LPC (17:1)[1]	0.34	508.3[*] / 530.3[#]
SM (d18:1/17:0)[1]	2.44	717.6[*] / 739.6[#]
PC (17:0/17:0)[1]	1.03	762.6[*] / 784.6[#]
LPA (17:1)[2]	1.80	421.2
LPS (13:0)[2]	8.80	454.2
LPE (17:1)[2]	4.23	464.3
LPG (17:1)[2]	1.77	495.3
LPI (17:1)[2]	1.35	583.3
PA (16:0/18:1)[2]	6.31	673.5
PS (14:0/14:0)[2]	8.74	591.5[§] / 678.5
PE (17:0/20:4)[2]	3.95	752.5
PG (17:0/20:4)[2]	1.35	783.5
PI (21:0/22:6)[2]	1.11	951.6

1-2. External ionization standards (ionization STDs)

Standards	Concentration (μM)	Detected *m/z*
PC (14:0/14:0)[1]	1.48	678.5[*] / 700.6[#]
PG (17:0/14:1)[2]	1.39	705.5

[1] Detected in positive mode.
[2] Detected in negative mode.
[*] H^+ adduct.
[#] Na^+ adduct.
[§] Loss of serine head group from PS.

2.2. Urine collection

This study was approved by the Ethics Committee of the Kyoto University Graduate School of Medicine and Kyoto University Hospital (Approval No. G52). Informed consent was obtained from each of the participants prior to sample collection. The urine samples were collected from males over 50 years old. The samples were centrifuged at 2000 × *g* for 10 min at 20 °C. The supernatants were filtered through 100 μm nylon cell strainers (BD Falcon, San Jose, CA, USA) to exclude urinary debris. Each filtered supernatant (1 mL) was placed in a 1.5 mL Flex-Tube® 3810X (Eppendorf, Hamburg, Germany) and stored at −80 °C until analyzed.

2.3. Mixed lipid internal standard solutions and external ionization standards

An internal standard (internal STD) mix containing 13 lipids was prepared in a 10 mL glass tube with 1.7 nmol LPC (17:1), 12.2 nmol SM

(d18:1/17:0), 5.15 nmol PC (17:0/17:0), 9.0 nmol LPA (17:1), 44.0 nmol LPS (13:0), 21.2 nmol LPE (17:1), 8.85 nmol LPG (17:1), 6.75 nmol LPI (17:1), 31.6 nmol PA (16:0/18:1), 43.7 nmol PS (17:0/14:1), 19.8 nmol PE (17:0/20:4), 6.75 nmol PG (17:0/20:4), and 5.55 nmol PI (21:0/22:6). The mixture was dried under N_2 gas, completely reconstituted in 5 mL $CHCl_3$:MeOH (1:1, v/v) to obtain a uniform and stable solution, and stored at −30 °C until used. The final concentrations of the internal STDs are shown in Table 1. The external ionization STDs (ionization STDs) were diluted in $CHCl_3$:MeOH:H_2O (60:30:4.5, v/v/v) to final concentrations of 1.48 μM PC (14:0/14:0) and 1.39 μM PG (17:0/14:1). These internal and ionization STDs were chosen because they were undetected or detected at very low concentrations in urine. As the ionization STDs, we chose PC (14:0/14:0) in the positive mode and PG (17:0/14:1) in the negative mode. The ionization STDs were used to normalize the peak intensities of the internal STDs. The peak intensities of each normalized internal STD showed a high correlation coefficient when plotted as a function of their corresponding concentrations. The composition and concentrations selected for our internal STD mix were modified slightly from the lipid internal standards used in a previous urinary lipidomic study (Rockwell et al., 2016). PS was the most abundant lipid species in the mix, based on the recently published urinary lipidomic research (Graessler et al., 2018). The optimized ionization STD concentrations was varied from 20% to 650% of the internal STD concentrations, based on a previous study (Szájli et al., 2008), to ensure linear responses during the MALDI-TOF/MS quantitative analysis. The concentration ranges with a linear response in our experiment were found to be as follows: 0.02–0.34 μM for LPC; 0.15–2.44 μM for SM; 0.06–1.03 μM for PC; 0.11–1.80 μM for LPA; 0.55–8.80 μM for LPS; 0.26–4.23 μM for LPE; 0.11–1.77 μM for LPG; 0.08–1.35 μM for LPI; 0.39–6.31 μM for PA; 0.55–8.74 μM for PS; 0.25–3.95 μM for PE; 0.08–1.35 μM for PG and 0.07–1.11 μM for PI.

2.4. Matrix preparation

9-AA (10 mM) was dissolved in 60:40 (v/v) isopropanol:ACN. DHB (65 mM) was dissolved in 100% MeOH. After thorough mixing, the solutions were centrifuged at 15,000 × g for 3 min, and the supernatants were transferred to a new tube. Lipid extract (3 μL) was pipetted into a new 0.5 mL Eppendorf safe-lock microtube and mixed with 3 μL of the matrix solution. The final mixture (1 μL) was spotted onto a μFocus MALDI plate (384 circles, 700 μm spot size) from Hudson Surface Technology, Inc. (Fort Lee, NJ, USA) and allowed to dry at room temperature. The μFocus plate is a disposable MALDI plate with a small hydrophilic island located in the middle of each well and surrounded by hydrophobic regions. When the sample solution is placed onto the μFocus spot, the hydrophobic and hydrophilic patterning causes the sample droplets to center upon the hydrophilic spot, avoiding the surrounding lipophilic area. The hydrophobic and hydrophilic patterning controls the deposition of the matrix crystals and analyte so that a small amount of sample is required, and the entire analyte sample is deposited into a small, controlled region on the plate. The small μFocus spots and correspondingly small amount of matrix yield optimum matrix/analyte ratios, allowing a good sensitivity with minute amounts of analyte. In a previous study, the efficiency of analyte enrichment with the μFocus plate was 1.5–2.5 times greater than that with the metal plate (Wang et al., 2011).

2.5. MALDI-TOF/MS analysis

Mass spectra were acquired over the *m/z* range 400–1000 in an Axima Performance MALDI-TOF/MS (Shimadzu Kratos Analytical, Manchester, UK). LPC, SM, and PC were detected in the positive reflectron mode, while LPA, LPS, LPE, LPG, LPI, PA, PS, PE, PG, and PI were detected in the negative reflectron mode. The power of the 337 nm nitrogen UV laser was adjusted so that it was just above the ionization threshold of the sample. The laser frequency was set to 10 Hz with 100 laser shots per profile, and 253 profiles were averaged for each individual sample. Argon gas was used for fragmentation by collision-induced dissociation (CID) at a collision energy (CE) of 20 keV. The LIPID MAPS® (URL: https://www.lipidmaps.org) and the SimLipid® version 6.0.5 software package (PREMIER Biosoft International, CA, USA) were used to identify the urinary phospholipids. The *m/z* values of the lipid ions were searched in the LIPID MAPS structure database (LMSD; URL: https://www.lipidmaps.org/tools/ms/LMSD_search_mass_options.php). $[M+H]^+$ and $[M+Na]^+$ ions were selected for the positive mode, while $[M-H]^-$ was selected for the negative mode. The mass tolerance was set at ± 0.2 Da. The lipid categories were limited to glycerophospholipids (GPLs) and sphingolipids (SPs). The MS/MS analysis was performed on the peaks of interest using the CID mode. The SimLipid® software was used to analyze the MS/MS spectra for the identification of the GPLs and SPs. All the matched precursors were scored based on the number of matched fragments in the MS/MS spectra. The species with the highest score was selected as the identified lipid.

2.6. Evaluation of MALDI matrices for lipid analysis

A schema of the protocol used for MALDI matrix evaluation is shown in Fig. 1A. The MALDI matrices 9-AA and DHB were compared in both positive and negative modes. The internal STD mix (10 μL) and the ionization STDs (1 μL) were added to the matrix solution, and the

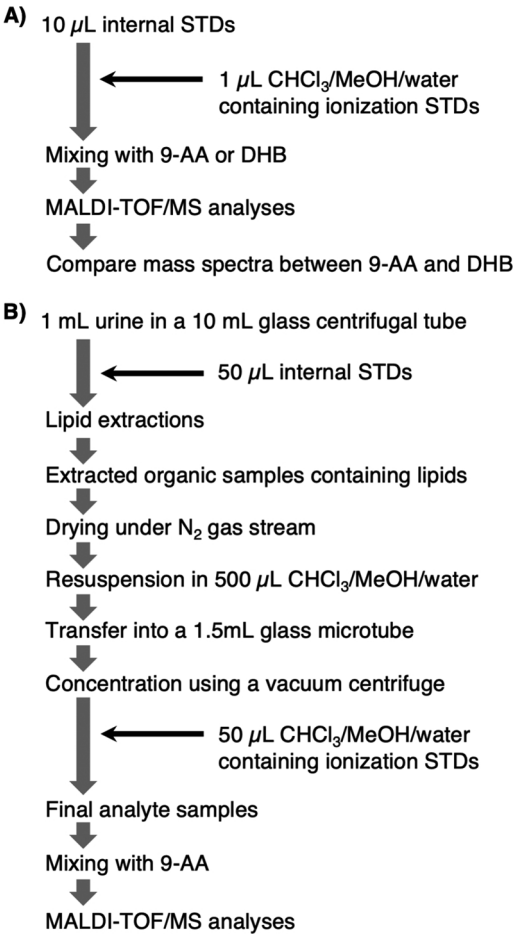

Fig. 1. Protocol schemas for matrix test (A) and analysis of extracted lipids (B). The internal STDs contained 13 lipid species. PC (14:0/14:0) and PG (17:0/14:1) were used as the external ionization STDs in positive and negative modes, respectively, for correction and normalization of the lipid ionization rates. The 9-AA and DHB indicated 9-aminoacridine and 2,5-dihydroxybenzoic acid, respectively.

mixture was applied to the μFocus MALDI plate and allowed to dry at room temperature. The number of detected lipid species and their peak intensities were used to evaluated the lipid ionization efficiency of the MALDI matrix. The *m/z* of each internal STD and ionization STD species is shown in Table 1.

2.7. Lipid extractions

The protocol schema for lipid extraction and analysis is shown in Fig. 1B. After thawing at room temperature, the 1 mL urine samples were centrifuged at 2000 × *g* for 5 min at room temperature. Each urinary supernatant was transferred to a 10 mL glass centrifuge tube to remove insoluble particles and used for analysis of urinary species. The internal STD mix (50 μL) was spiked into each urine sample and mixed well. Lipid extractions were performed in triplicate using the six different solvent systems described in subsections 2.7.1–2.7.6. After each extraction, the organic phase containing the extracted lipids was dried under an N_2 stream and resuspended in 500 μL $CHCl_3$:MeOH:H_2O (60:30:4.5, v/v/v). Each resuspended sample was transferred to a F-MGT-1.5 1.5 mL glass microtube (Fujirika Kogyo Co., LTD., Osaka, Japan) and concentrated in a CVE-3100 centrifugal evaporator (EYELA, Tokyo, Japan) equipped with a Unitrap UT-2000 cold trap. The concentrated sample was reconstituted in 50 μL of the ionization STD mix described in section 2.3 and used for MALDI-TOF/MS analyses.

2.7.1. Lipid extraction by Folch method

According to Folch method (Folch et al., 1957), following the addition of 5 mL ice-cold $CHCl_3$:MeOH (2:1, v/v), each sample mixture was vortexed for 10 min. Ultrapure water (0.25 mL) was then added to the mixture, which was vortexed for an additional 10 min. The sample was centrifuged at 2000 × *g* for 10 min at room temperature, and the lower (organic) phase was transferred to a clean glass tube with a glass Hamilton syringe (Bonaduz, GR, Switzerland). The remaining aqueous phase was re-extracted with 2.5 mL ice-cold $CHCl_3$ using the same procedure. The lower phase was collected and added to the first extract. The combined organic phase was washed with 2 mL water and dried in a centrifugal evaporator.

2.7.2. Lipid extraction by Bligh and Dyer method

According to Bligh and Dyer (B&D) method (Bligh and Dyer, 1959), following the addition of 3.75 mL ice-cold $CHCl_3$:MeOH (1:2, v/v), each sample mixture was vortexed for 10 min. Ice-cold $CHCl_3$ (1.25 mL) was added and mixed for 5 min. Water (1.25 mL) was then added with an with continuous mixing for 5 min. The mixture was centrifuged for 10 min at 2000 × *g*, and the lower (organic) phase was transferred to a clean glass tube with a glass Hamilton syringe. Ice-cold $CHCl_3$ (1.88 mL) was added to the aqueous residue to extract the remaining lipids. The organic phases were combined, washed with 2 mL water, and dried in a centrifugal evaporator.

2.7.3. Lipid extraction by Acidified Bligh and Dyer method

Lipids were extracted according to the B&D method (Retra et al., 2008; Reis et al., 2013), but 30 μL 6 M HCl was added following the addition of 1.88 mL ice-cold $CHCl_3$ for extraction of the aqueous residue.

2.7.4. Lipid extraction by methyl-tert-butyl ether method

According to the methyl-tert-butyl ether (MTBE) method (Matyash et al., 2008), following the addition of 6.5 mL ice-cold MTBE:MeOH (10:3, v/v), each sample mixture was vortexed for 10 min. Ultrapure water (0.25 mL) was then added to the mixture, which was vortexed for another 10 min. The sample was centrifuged for 10 min at 2000 × *g*, and the upper (organic) phase was transferred to a clean glass tube with a glass Hamilton syringe. Extraction of the aqueous bottom layer was performed by adding 2 mL MTBE:MeOH:H_2O (10:3:2.5, v/v/v) and incubating for 15 min, followed by centrifugation at 2000 × *g* for 10 min. The organic phases were combined, washed with 2 mL water, and dried in a centrifugal evaporator.

2.7.5. Lipid extraction by improved butanol/methanol method

According to the improved butanol/methanol (BUME) method (Löfgren et al., 2012; Cruz et al., 2016), following the addition of 3 mL ice-cold butanol:MeOH (3:1, v/v), each sample mixture was vortexed for 10 min. A 3:1 (v/v) solution of heptane:ethyl acetate (3 mL) was added, and the mixture was vortexed for another 10 min. A 50 mM LiCl solution (3 mL) was added to the tube, and the mixture was vortexed for an additional 10 min. Following centrifugation for 10 min at 2000 × *g*, the upper (organic) phase was transferred to a clean glass tube. The aqueous phase was re-extracted with 3.2 mL heptane:ethyl acetate (3:1, v/v), and the organic phases were combined. The combined organic phase was washed with 2 mL water and dried in a centrifugal evaporator.

2.7.6. Lipid extraction by hexane/isopropanol method

According to the hexane/isopropanol (HIP) method (Hara and Radin, 1978), following the addition of 5 mL ice-cold hexane:isopropanol (3:2, v/v), the mixture was vortexed for 10 min. Ultrapure water (2.3 mL) was added, followed by mixing for an additional 10 min. The mixture was centrifuged at 2000 × *g* for 10 min, and the upper (organic) phase was transferred to a clean glass tube with a glass Hamilton syringe. The lower aqueous phase was re-extracted with 2 mL hexane:isopropanol (3:2, v/v) for 15 min by vortex mixing. After the final centrifugation, both the organic phases were combined, washed with 2 mL water, and dried in a centrifugal evaporator.

2.8. Normalization of target peak intensities using external ionization standards

The absolute peak intensities in a MALDI-TOF/MS spectrum may vary significantly among different shots of the same sample or between wells (Duncan et al., 2008). Owing to this, all the mass spectral data collected in this study were normalized to the ionization STD data. The ionization STDs were selected so that their *m/z* values lay in the mid-range of those of the analytes (Table 1), and the PC (14:0/14:0) and PG (17:0/14:1) concentrations did not vary among wells in the MALDI plate. For analysis in the positive mode, the intensities of the target LPC, SM, and PC peaks were normalized using the PC (14:0/14:0) peaks at *m/z* 678.5 and *m/z* 700.6. In the negative mode, the peak intensities of the negatively charged ionized lipids (LPA, LPS, LPE, LPG, LPI, PA, PS, PE, PG, and PI) were normalized using the PG (17:0/14:1) peak at *m/z* 705.5. The mass spectral data were processed with OpenMS (Röst et al., 2016). After noise filtering, baseline subtraction, and peak picking, the data were exported in Excel (Microsoft Corp., Redmond, WA, USA) as CSV files to calculate the relative peak intensities. The intensity of each target peak in the spectrum was first normalized with respect to each ionization STD by calculating the ratio of the target peak intensity to ionization STD peak intensity. The normalized peak intensities were then used to determine the lipid concentrations in the MALDI-plate wells.

2.9. Reliability of quantitative lipid analyses by MALDI-TOF/MS

The standard calibration curves were prepared by serial dilution of the internal STDs. The internal STD mix was diluted to 6.25%, 12.5%, 18.75%, and 25% of the original concentration in $CHCl_3$:MeOH (1:1, v/v). $CHCl_3$:MeOH (1:1, v/v) with no internal STD was used as the 0% calibration standard. The solutions were prepared with fixed concentrations of the ionization STDs and analyzed by MALDI-TOF/MS on three different days. Standard curves for each lipid were constructed by plotting nominal lipid concentrations against the normalized lipid peak intensities (section 2.8). The evaluation of the coefficient of determination (R^2) of each curve gave an estimate of the linearity and

reliability of MALDI-TOF/MS for quantitative analysis.

2.10. Examining lipid recovery with six different lipid extraction methods

To determine the lipid recovery for each extraction method described in section 2.7, solutions were first prepared to reflect 100% recovery. Internal STD mix (50 µL) was added to a 10 mL glass tube, dried under streaming N_2, and re-suspended in 500 µL $CHCl_3$:MeOH:H_2O (60:30:4.5, v/v/v) without extraction. The re-suspended mixture was transferred to a 1.5 mL glass microtube and concentrated in a centrifugal evaporator. The concentrated sample was reconstituted in 50 µL $CHCl_3$:MeOH:H_2O (60:30:4.5, v/v/v) containing the ionization STDs. The 9-AA matrix was added to each sample and spotted for MALDI-TOF/MS analysis. The plates were analyzed on three different days. The relative signal intensity of each internal STD following extraction was compared with the relative intensity of the corresponding peak representing 100% recovery, in order to determine the lipid recovery. The extracted samples and 100% recovery group prepared on a given day were analyzed in the same MALDI-TOF/MS run to reduce analytical variation between runs.

2.11. Detection of urinary lipids using our screening method

Endogenous urinary lipids were analyzed for validating the optimized extraction and analysis protocol. Urine samples of three male patients suffering from lower urinary tract disease were used for these validation tests. One milliliter urine without any internal standards was extracted by the acidified B&D method, the organic extract was dried under nitrogen stream, and finally resuspended in 200 µL of $CHCl_3$/MeOH/water mixture (60:30:4.5, v/v/v). Equal volumes of the extraction solution were mixed with 10 mM 9-AA dissolved in isopropanol/ACN (60:40, v/v). The dried mixture was analyzed in both positive and negative mode for screening the endogenous urinary lipids. The identification of urinary lipids is described in section 2.5.

2.12. Statistical analyses

Statistical analyses were performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA) or JMP® Pro 13.2.0 (SAS Institute, Cary, NC, USA) licensed to Kyoto University. All the values are expressed as mean ± standard deviation (SD).

3. Results and discussion

3.1. Roles of internal and ionization standards in MALDI analysis

In this study, 15 commercially available lipids were chosen for preparing internal and ionization STD solutions (Table 1). The internal STDs and ionization STDs are listed in Table 1-1 and 1-2, respectively. The ion suppression of PE by PC in the mixture hindered the quantitative evaluation of PE, because of which we analyzed PE in the negative mode. However, PE is a zwitterionic phospholipid and could also be detected in the positive mode (Fuchs et al., 2010). The ionization STDs were selected to account for the variable ionization efficiency of MALDI-TOF/MS, because the absolute signal intensities did not reflect the linearity of sample concentrations between different wells. Thus, a normalization step was necessary for data processing following the analysis, in which the ionization STDs were used for correction.

The internal STD mix prepared for this study comprised 13 unlabeled, commercially available lipids. Representative mass spectra of the internal STDs are shown in Fig. 2. Avanti Polar Lipids, Inc. recently developed a deuterated standard mixture called EquiSPLASH™ LIPIDOMIX® Quantitative Mass Spec Internal Standard (https://avantilipids.com/product/330731). This STD mixture consists of 13 deuterated lipids at a concentration of 100 µg/mL each, which have been verified for use as quantitative standards. Unfortunately, we were not able to purchase the new STD at the time of submission, but the EquiSPLASH™ LIPIDOMIX® might be a convenient and economical tool for quantitative MALDI-TOF/MS analysis.

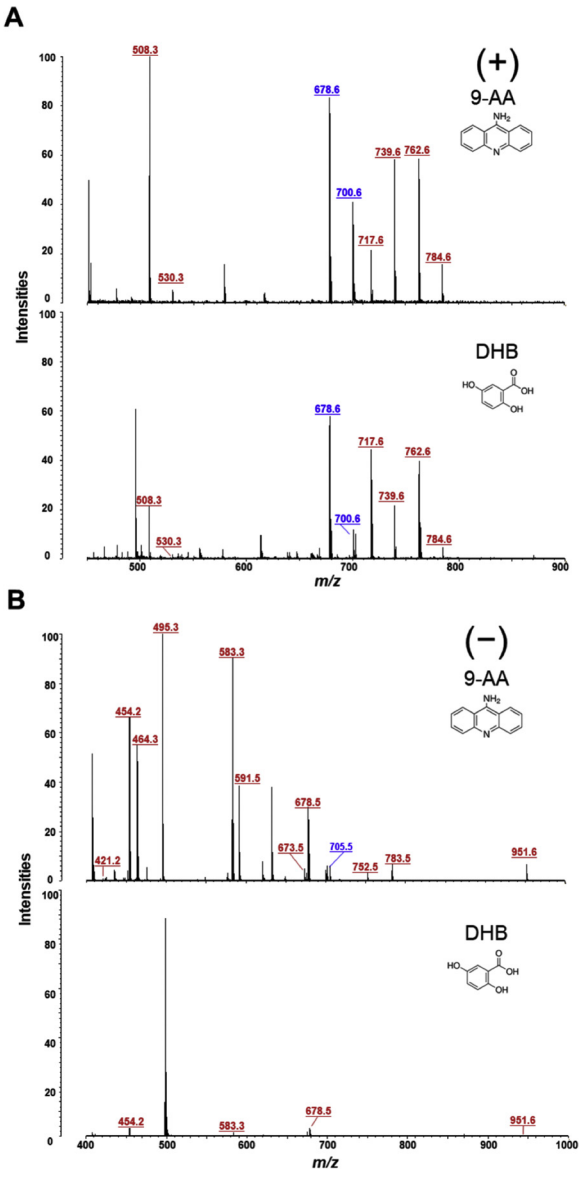

Fig. 2. Representative mass spectra of the internal and ionization standards. Spectra in A and B were collected in positive and negative modes, respectively. The internal STDs LPC, SM, and PC were detected in the positive mode. LPA, LPS, LPE, LPG, LPI, PA, PS, PE, PG, and PI were detected in the negative mode. The *m/z* ratios of the internal STDs and external ionization STDs are shown in red and blue, respectively. LPC, SM, and PC were detected in both 9-AA and DHB matrices in the positive mode. All 10 lipid species were detected in 9-AA in the negative mode, but only LPS, LPI, PS, and PI were detected in DHB. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.2. Optimization of MALDI matrix in positive and negative ionization modes

The commonly used MALDI matrices 9-AA and DHB were compared as shown in Fig. 1A. The number of matrix peaks and their intensities were used to evaluate lipid ionization efficiency in each matrix in both positive and negative modes. Representative mass spectra of the ionization STDs are shown in Fig. 2. Among the internal STDs analyzed in the positive mode, LPC was detected as H^+ (*m/z* 508.3) and Na^+ (*m/z*

530.3) adducts. The H^+ (*m/z* 717.6) and Na^+ (*m/z* 739.6) adducts of SM were also detected, as were the H^+ (*m/z* 762.6) and Na^+ (*m/z* 784.6) adducts of PC. PC (14:0/14:0) was detected as H^+ (*m/z* 678.6) and Na^+ (*m/z* 700.6) adducts as well. All the internal and ionization STDs were detected in both 9-AA and DHB matrices in the positive mode in this study. However, the signal-to-noise (S/N) ratios in the spectra of the LPC, SM, and PC targets in the 9-AA matrix were higher than those observed in DHB, which was in agreement with the results of a previous study (Sun et al., 2008). Moreover, a strong peak at *m/z* 498.8 was identified as the DHB matrix cluster, while background peaks (e.g. *m/z* 579.4) of lower intensities were attributed to 9-AA.

Remarkable differences between 9-AA and DHB were observed during MALDI-TOF/MS analysis in the negative mode. In the 9-AA matrix, all the 10 internal STDs [LPA (*m/z* 421.2), LPS (*m/z* 454.2), LPE (*m/z* 464.3), LPG (*m/z* 495.3), LPI (*m/z* 583.3), PA (*m/z* 673.5), PS (*m/z* 678.5), PE (*m/z* 752.5), PG (*m/z* 783.5) and PI (*m/z* 951.6)] were detected. One ionization STD [PG (17:0/14:1) at *m/z* 705.5] was also detected. The peak at *m/z* 591.5 indicated the loss of serine head group of PS by in-source decay. All the lipids were observed in the 9-AA matrix; however, only four internal STDs [LPS (*m/z* 454.2), LPI (*m/z* 583.3), PS (*m/z* 678.5), and PI (*m/z* 951.6)] were detected in the DHB matrix. The other seven lipids, comprising six internal and one ionization STD, were not detected in the DHB matrix.

Although the DHB matrix has a lower proton affinity compared with the 9-AA matrix, there was a minor difference in the positive ion spectra of the 9-AA and DHB matrices in our experiments. This was similar to the findings of some previous studies (Leopold et al., 2018; Sun et al., 2008). However, we chose 9-AA, and not DHB, as the matrix for the positive mode because of two reasons. The first reason was that 9-AA could result in more even analyte distribution as compared with DHB, which formed sweet spots in the crystalline matrix needles. The second reason was that 9-AA resulted in low background in the negative mode (as compared with DHB), offering a more reproducible quantitative analysis among the sample spots in the negative mode (Sun et al., 2008; Wang and Giese, 2017). The advantages of 9-AA over DHB were also reported in the PC quantitation in the positive mode (Chagovets et al., 2015), in the generation of abundant H^+ adducts, and in the moderate production of Na^+ adducts, which enabled simplified peak assignment during the analysis of complex mixtures (Pyttel et al., 2012). Therefore, 9-AA has been identified as one of the most suitable and effective matrices for the analysis of urinary lipids.

The appropriate analyte-to-matrix ratio, which was determined from the concentrations of 9-AA and total lipids extracted from urine in this study, is very important in MALDI-TOF analyses (Wang and Giese, 2017). This is because the total lipid concentration in urine is much lower than that in plasma, tissues, or cells. The concentration of 9-AA should be evaluated to identify the proper analyte-to-matrix ratio for high sensitivity and low matrix background. When 9-AA was prepared at 10 mg/mL (51.5 mM) in 60:40 (v/v) isopropanol:ACN (Sun et al., 2008), several strong background peaks at *m/z* 579.2 and *m/z* 595.2 were detected in the positive mode and were found to originate from the 9-AA matrix cluster (Fig. 3). After reducing the concentration of 9-AA from 50 to 10 mM, the intensities of these matrix-generated peaks were reduced to barely detectable levels. At the same time, the S/N ratios for the detection of lipids were improved. Therefore, we found that 9-AA concentrations ≤ 5 mM was proportionate for measuring lipid concentrations in 1 mL urine samples in the 700-μm μFocus MALDI plates.

3.3. Quantitative MALDI analysis with internal STD standard curves

The standard curves constructed for each of the internal STDs are shown in Fig. 4. The STD curves in Fig. 4A and B were plotted using the results obtained in the positive and negative modes, respectively. Each R^2 value is the coefficient of determination between the relative peak intensity ratios (i.e. the intensity ratios between each internal STD and the ionization STD intensities) and concentrations of the spiked target lipids. LPG yielded the highest R^2 of 0.969, while PE had the lowest R^2 of 0.907. The R^2 values ranging from 0.907 to 0.969 indicated a strong linear correlation for all the lipids. The linear responses observed with all the lipids thus indicated our analytical procedures were suitable for semi-quantitative and comprehensive screening of PLs and LPLs by MALDI-TOF/MS.

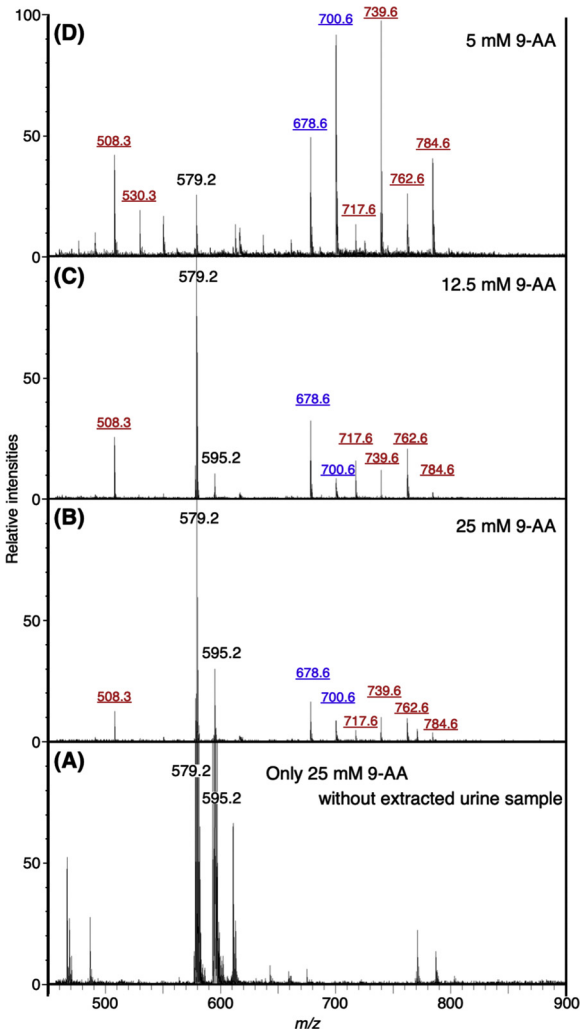

Fig. 3. Representative mass spectra of extracted urine samples spiked with internal and ionization STDs at different 9-AA concentrations. All samples were analyzed in the positive mode. Total ion current intensities in the mass ranges shown were 14 mV. (A) 25 mM 9-AA matrix solution without any lipid species. The peaks at *m/z* 579.2 and *m/z* 595.2 originated from 9-AA. Spectra of 9-AA at final concentrations of 25 mM (B), 12.5 mM (C), and 5 mM (D) mixed with 1 mL urine spiked with internal and ionization STDs. According to the method describe in Fig. 1B, the urine sample spiked with the internal STDs was extracted with acidified B&D method and analyzed. The Internal STD *m/z* ratios are shown in red, and ionization STD *m/z* ratios are shown in blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.4. Evaluation of extraction methods from mass spectra of urine samples spiked with internal STDs

Representative mass spectra of extracts obtained with each of the extraction methods are shown in Fig. 5. The spectra of extracts analyzed in the positive and negative modes can be seen in Fig. 5A and B, respectively. In the positive mode, the average PC (14:0/14:0) peak intensities at *m/z* 678.6 and *m/z* 700.6 were set to approximately 70% of

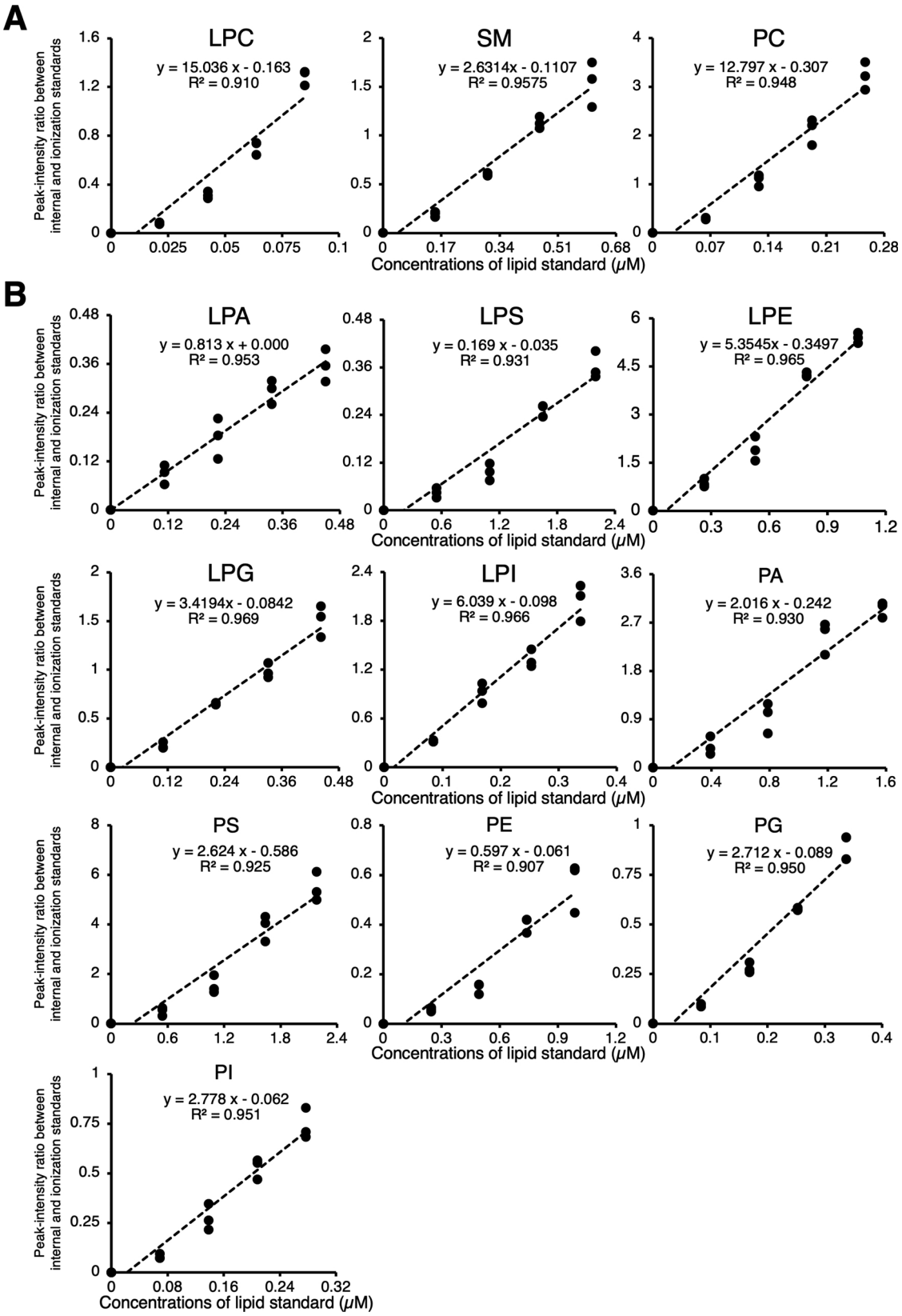

Fig. 4. Standard curves from quantitative MALDI analysis with internal STDs. The data shown in A and B were collected in the positive and negative modes, respectively. Coefficient of determination (R^2) was calculated from the curve and evaluated to investigate the reliability of MALDI-TOF/MS for the quantitative analysis. Strong linear correlations were revealed between the normalized intensity and concentration of each lipid subclass, with R^2 values ranging from 0.907 to 0.969.

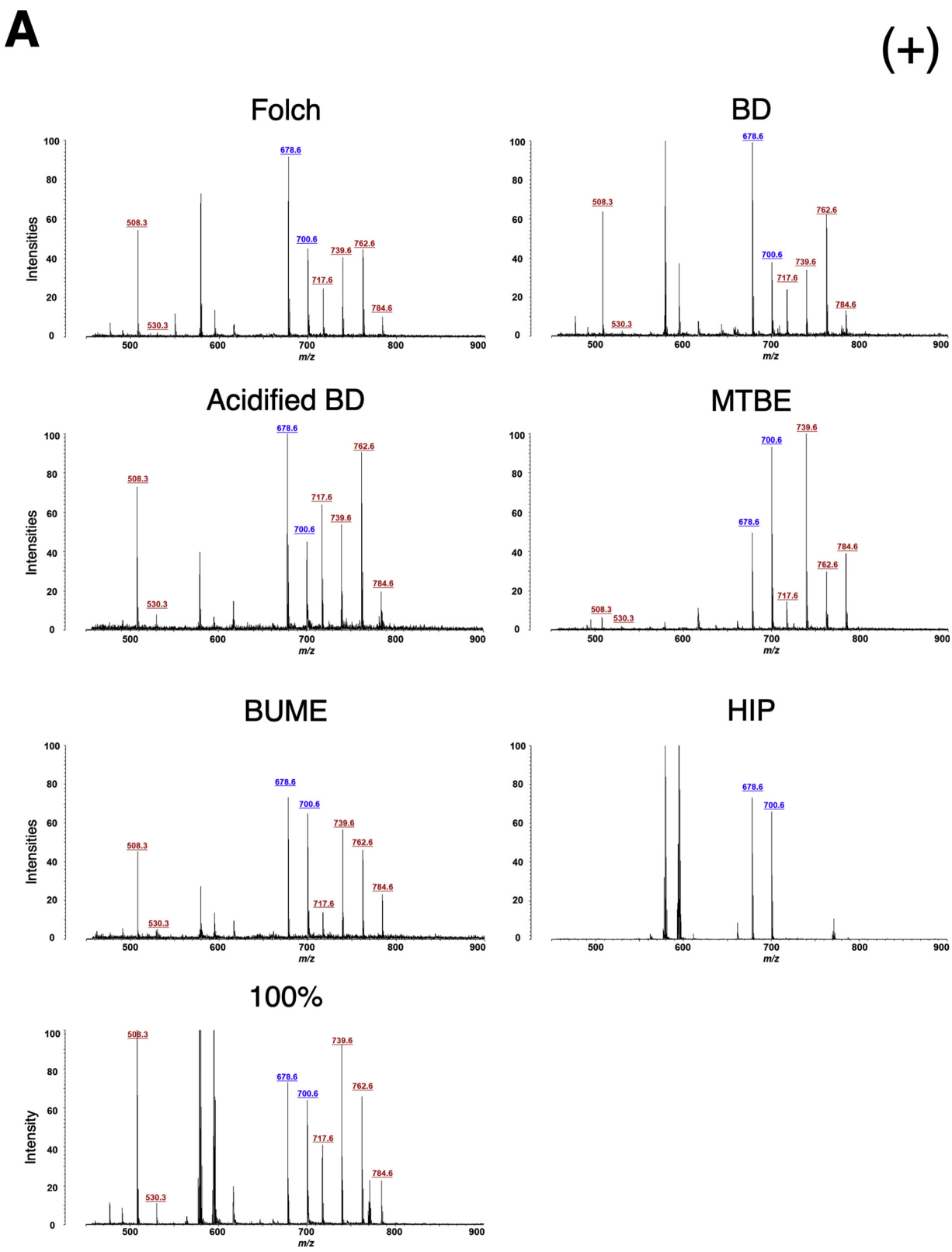

Fig. 5. Representative mass spectra of spiked urine samples in the 9-AA matrix. Data shown in A and B were collected in the positive and negative modes, respectively. Internal STD *m/z* ratios are shown in red, and ionization STD *m/z* ratios are shown in blue. In the positive mode, the average PC (14:0/14:0) peak intensities at *m/z* 678.6 and *m/z* 700.6 were set to approximately 70% of the maximum on the y-axis. In the negative mode, the peak intensities at *m/z* 705.5 originating from the ionization of PG (17:0/14:1) were set to approximately 10% of the maximum on the y-axis. All internal STDs were detected only in the acidified B&D extracts. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

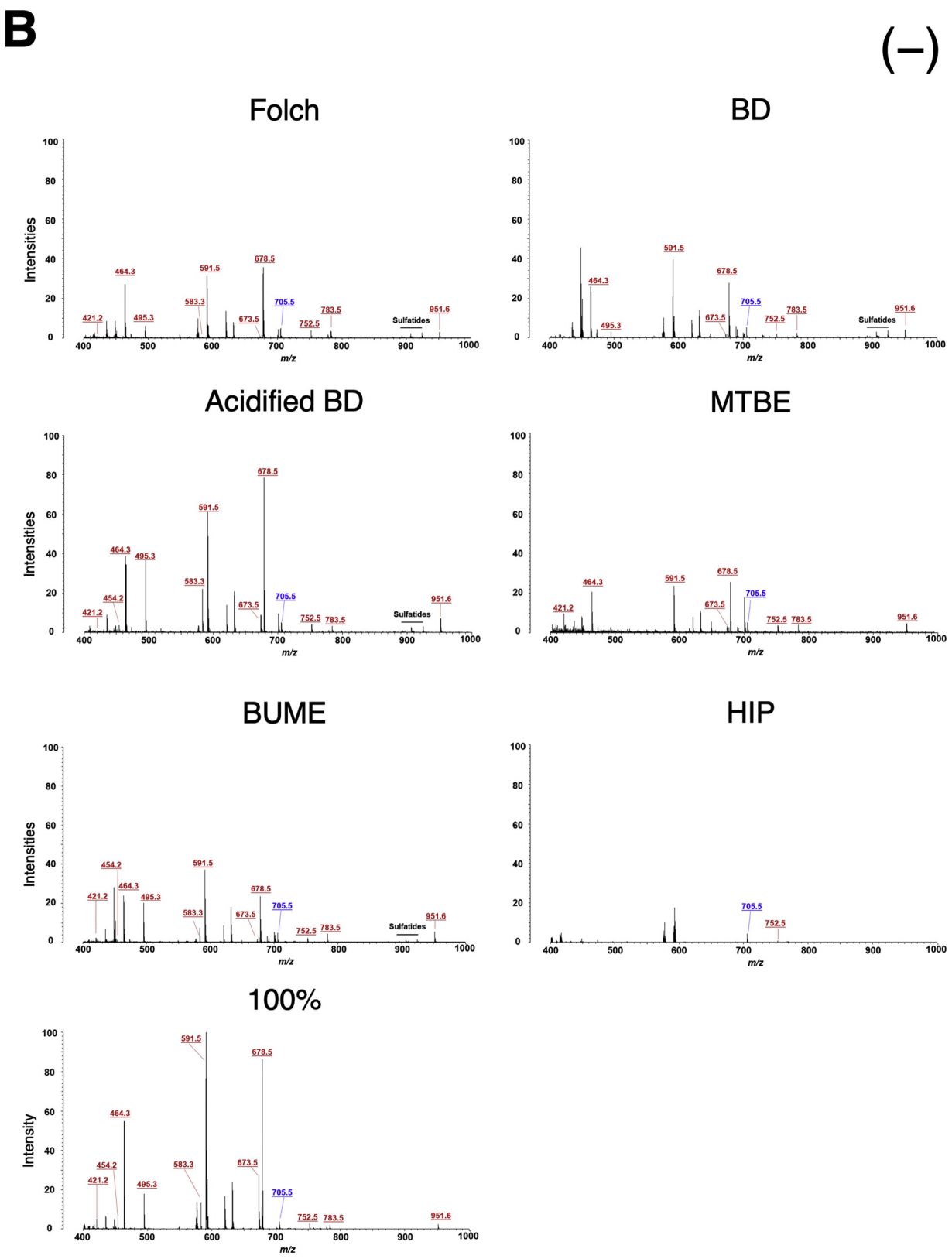

Fig. 5. (*continued*)

the maximum on the y-axis. Recoveries of LPC, SM, and PC were high with the Folch, B&D, and acidified B&D methods, which employed $CHCl_3$:MeOH for extraction. However, with the MTBE method, a much smaller amount of LPC compared with either SM and PC, was extracted, while the BUME method had a lower recovery efficiency for SM compared with LPC and PC. Recoveries of all the three species were poor with the HIP method. Among these five extraction methods, the acidified B&D method was the most efficient for the extraction of LPC, SM, and PC, and recoveries with the acidified B&D method were similar to previously reported values (Reis et al., 2013). In the report by Reis

et al. (2013), the effectiveness of the five different solvent systems was compared by extracting lipids from LDL particles isolated from human plasma. The researchers concluded that the most suitable solvent systems for LDL extraction were those used in the Folch and acidified B&D methods. A more detailed comparison of the recoveries from the five published extraction methods is discussed in the next section.

In the negative mode, the peak intensity at *m/z* 705.5 originating from the ionization of PG (17:0/14:1) was set to approximately 10% of the maximum on the y-axis. The HIP extraction method was not effective, because PE was the only lipid species from the internal STD mix detected in the extracts. None of the other nine species were detected, so we excluded the HIP method from subsequent comparisons. The only method that enabled highly sensitive detection of all the internal STDs was the acidified B&D method. In a comparison with the peak intensities of the ionization STD, the intensities of the LPA, LPE, LPG, LPI, PA, and PS peaks from the acidified B&D extracts were the highest among the methods. However, the PE peak intensity was very low in the extracts from all the five methods. The best extraction method for PE was the MTBE method.

Small peaks at *m/z* 701.5 were observed in the spectra of the samples extracted by the Folch, B&D, acidified B&D, MTBE, and BUME methods. The same peak was also detected in the spectrum in the negative mode with 9-AA matrix (Fig. 2B). This peak was thought to originate from the SM (d18:1/17:0) and PC (17:0/17:0) ions contained in the internal STDs, as shown in supplementary Fig. 1. The internal STDs including only LPC (17:1), SM, and PC were analyzed in both positive and negative modes. The existence of the demethylated ions of LPC, SM, and PC, with the loss of *m/z* 16 (CH_4) compared with positive mode, were confirmed. The demethylated ions of LPC, SM, and PC was detected at *m/z* 492.3, 701.5, and 746.5, respectively. Several fragmented ions with the loses of *m/z* 61 and 87 were also observed, as described in previous reports (Fuchs et al., 2009; Leopold et al., 2018). The estimated chemical structures of the loose ions are shown in the supplementary Fig. 1. The colocalization of the demethylated SM and the PC with dissociated *m/z* 61 was confirmed as the fragment PC peak was observed at *m/z* 701.5. In our experiments, the other peaks, except for those at *m/z* 701.5, were not detected when mixed with other PLs and LPLs and when extracted from urine. We speculate that the phenomenon was suppressed under the experimental conditions owing to the presence of a large amount of lipids in the endogenous bio-matrices of human urine samples.

Several sulfatides were detected near *m/z* 900 in our analysis. Sulfatides in human urine have been described as promising biomarkers of metachromatic leukodystrophy (MLD), a lysosomal storage disorder, in previous studies (Kuchař et al., 2013; Spacil et al., 2016). Sulfatides were also confirmed as one of the strong electrolytes, as over 35-fold selective desorption/ionization of sulfatide, as compared with anionic phospholipids, occurred using 9-AA as the matrix (Cheng et al., 2010). We have also observed peak clusters of urinary sulfatides with *m/z* values from 880.6 to 906.6. Some of the anionic phospholipid species [e.g. PI (18:0/20:4)] may be suppressed and overlapped because of the much higher desorption/ionization of sulfatides over PI. In the Folch and B&D extracts, the sulfatide peak intensities were high. However, their intensities were lower than those of PI in the acidified B&D and BUME extracts, indicating that these methods were suitable for detecting urinary PI. Thus, it was concluded that the acidified B&D method was the most suitable for extracting and analyzing urinary lipids using our protocol.

3.5. Evaluation of extraction methods based on recovery of spiked internal STDs from urine samples

Results of the recovery tests are summarized in Fig. 6. Data collected in the positive and negative modes are shown in Fig. 6A and B, respectively. Recoveries of LPC, SM, and PC were used to evaluate the six different extraction methods. Among these, the acidified B&D method was the most efficient for extracting LPC, SM, and PC. Recoveries with the acidified B&D method were 85.3% for LPC, 87.1% for SM, and 69.4% for PC. Three solvent systems consisting of mainly $CHCl_3$:MeOH in the Folch, B&D, and acidified B&D methods yielded relatively high recoveries for these three lipid species. PCs are studied most frequently by MALDI-TOF/MS analysis, since the permanent positive charge of their quaternary ammonium group is easily detected in the positive ion mode (Fuchs et al., 2010). PCs are the most abundant constituents of eukaryotic cellular membranes and are commercially available over a wide range (Gellermann et al., 2006). The MTBE extraction method was as effective as the acidified B&D method for SM, although an LPC recovery of only 10.7% indicated that it was not effective for the extraction of this species. The HIP method was not effective for the extraction of the urinary lipids analyzed in the positive mode, as recovery of each analyte was less than 5%.

Recoveries of the 10 species analyzed in the negative mode were lower than those of the species analyzed in the positive mode. Similar to that observed in the positive mode, the HIP method was not effective for nine of the urinary lipids analyzed in the negative mode, as recoveries were below 3%, with the exception of PE at 17.7%. The poor lipid recovery of the HIP method was described in a previous report (Reis et al., 2013). Therefore, the HIP method was excluded from all subsequent comparative analyses. Of the remaining five extraction methods, the best recovery was obtained with PS extracted by the acidified B&D method (85.3%). The lowest recovery was obtained with LPS extracted by the Folch method (12.7%). The acidified B&D method was the most effective among all the six extraction methods of LPLs. Recoveries of LPA, LPS, LPE, LPG, and LPI were 58.5%, 45.6%, 77.0%, 57.8%, and 63.7%, respectively. The acidified B&D method was also the most effective for extraction of the PLs, with recoveries of 64.0% (PA), 85.3% (PS), and 56.6% (PG). However, the acidified B&D method was the second best for PE and PI extraction. Among the six methods, the most effective for extraction of PE and PI were the MTBE and Folch methods, respectively. If only urinary PI species are considered, extraction with the Folch method could be recommended. LPS recovery was below 46.0% with all of the extraction methods, including the acidified B&D method. Thus the acidified B&D method was identified as the most suitable method for extraction in our comprehensive urinary lipid screening by MALDI-TOF/MS. Collectively, recovery of all lipid species with this method was higher than those with the other extraction methods.

Among the five methods still under consideration, the acidified B&D method was the most efficient for extraction of LPC, SM, PC, PE, and PI. These results were similar to those reported by Reis et al. (2013). They suggested that a preliminary study of the effectiveness of different solvent systems and data processing procedures prior to lipid analysis was important. Our results strongly support their conclusions, although they determined that both the Folch and acidified B&D methods were suitable for LDL analysis. We propose that the acidified B&D method might be the best option for extraction of urinary lipids.

It has been reported that the extraction efficiency of the acidified B&D method gradually increases as the number of double bonds in the fatty acid increases (Jensen, 2008). Portions of the fatty acids in the fecal samples were present in free form, some of which were difficult to extract due to tightly bound calcium ions. However, the acidified B&D method enabled efficient extraction of the free fatty acids bound with calcium ions (Jensen, 2008). Charged polar lipids, such as PLs and LPLs, can bind to various biomolecules through ionic interactions that cannot be easily disrupted by polar organic solvents. In such situations, adjusting the pH of the aqueous medium prior to extraction can be beneficial for the quantitative extraction. Acidification is an effective means for increasing the extraction efficiency. Acidification can convert negatively charged ionized lipids to their non-ionized forms, which interrupts ionic interactions and increases lipid hydrophobicity (Pati et al., 2016). This was demonstrated in a study in which the extraction of polar lipids from cells was improved by replacing water in the B&D

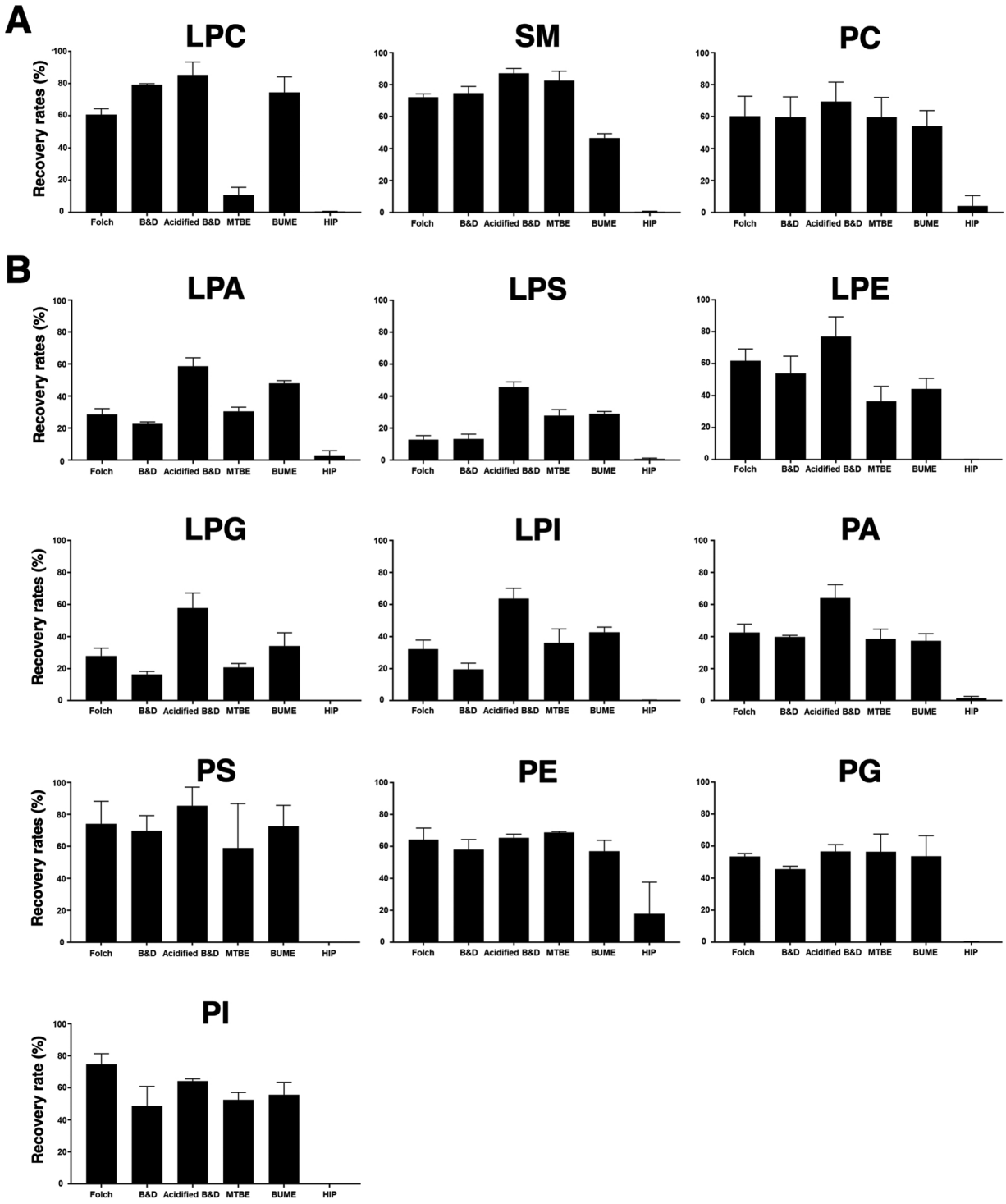

Fig. 6. Extraction recoveries of 13 internal STD lipid species extracted by 6 different methods. Data in A and B were collected in positive and negative modes, respectively. The relative recovery rates were calculated by comparing the normalized intensities of the urinary extraction groups and the 100% recovery groups. Mean recoveries are shown, and the bars indicate standard deviations (SD).

method with 5% TFA or 2 M HCl (Nishihara and Koga, 1987). Moreover, acidification can increase lipid ionization efficiency during mass spectrometry runs (Pati et al., 2016).

However, the presence of acid in the extraction solution could accelerate the hydrolysis of PL, increasing the LPL abundance, especially in plasmalogens. The hydrolysis is thought to be a time-dependent reaction (Pietruszko and Gray, 1962). In our experiments, all the solutions were maintained at 4 °C to suppress the reaction, and HCl was spiked into the water phase only in the second extraction step. There was another washing step using 2 mL water, after combining the organic phases. Therefore, the hydrolysis of PLs was thought to be negligible under our experimental conditions, because the total time under low pH was estimated to be within 1 h at room temperature. Moreover, in order to confirm our hypothesis, we extracted PC (17:0/17:0) solution using acidified B&D method with different HCl contact time (1, 3, 8, and 24 h, supplementary Fig. 2A). LPC (17:0) hydrolyzed from PC (17:0/17:0) were observed. The peak intensity ratios between LPC (17:0) and PC (17:0/17:0) were used to estimate the rates of hydrolysis

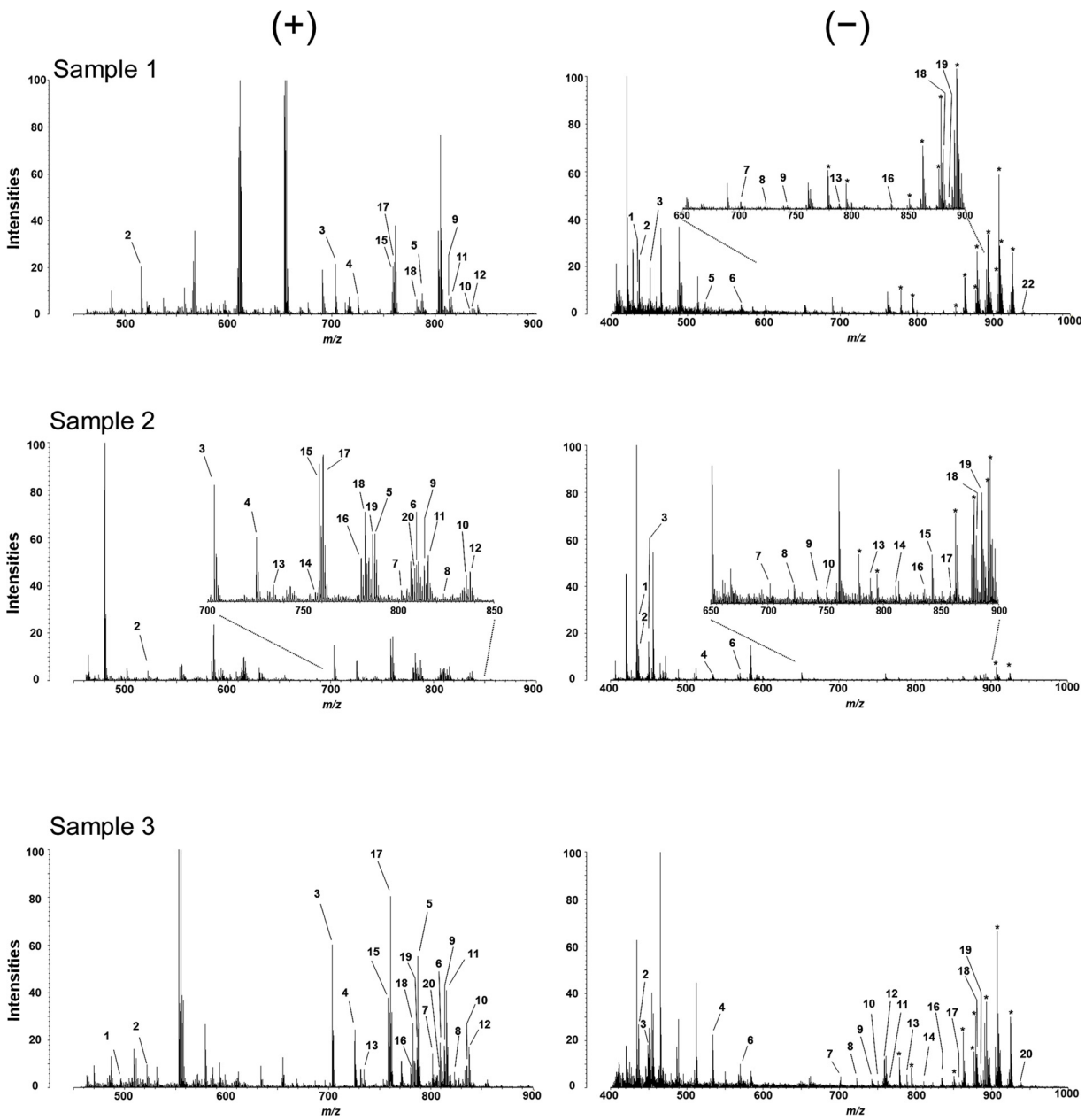

Fig. 7. Representative mass spectra of urinary lipid species analyzed by the optimized extraction and analysis protocol of our comprehensive lipid screening method. Urinary lipid species were extracted by the acidified B&D method and analyzed in both positive and negative mode using 5 mM 9-AA. Urine samples of three male patients (i.e., samples 1 to 3) suffering from lower urinary tract disease were used for these validation tests. A total of 11 and 20 lipids were detected in the positive and negative modes, respectively. The number at each peak of the spectra corresponds to the serial number of the lipid species in Table 2. The asterisk (*) indicates the peak cluster of urinary sulfatides.

(shown in supplementary Fig. 2B). The ratio in the absence of HCl was about 1.39%. The ratios in the presence of HCl in the extraction solution were also very low and were around 1% for 1, 3, and 8 h. Even at 24 h, the ratio was only about 1.43%. From the above results, we concluded that the ratio to produce LPC from PC was extremely low and exceedingly minor chemical reactions occurred in the acidified B&D method, which we had adopted for our analysis.

Therefore, we concluded that MALDI-TOF/MS analysis following acidified B&D method lipid extraction was suitable for the comprehensive screening of low concentrations of PLs and LPLs in small volumes of human urine samples, because the acid-catalyzed hydrolysis of PLs was negligible in our experiments.

3.6. Detection of urinary lipid species by our screening method

The optimized extraction and analysis protocol of our comprehensive lipid screening method was validated. Urine samples of three male patients suffering from lower urinary tract disease were used for these validation tests. In the three urine samples, a total of 31 lipid species were detected and identified by our screening method (Fig. 7, Table 2 and supplementary Fig. 3). The number at each peak of the spectra in Fig. 7 corresponds to the serial number of the lipid species in Table 2. In the positive mode, 11 lipid species, including 2 LPC, 5 SM, and 4 PC, were detected. LPCs were detected as only H^+ adducts, while SMs and PCs were detected as both H^+ and Na^+ adducts. In the negative mode, 20 lipid species, including 2 LPA, 2 LPE, 2 LPS, 1 PA, 4 PE, 4 PS, and 5 PI, were detected as deprotonated ions. Our optimized urinary lipid

Table 2
Detected urinary lipids using our comprehensive screening method.

2-1. Positive mode

No.	Lipid species	Detected *m/z*	Adduct ion
1	LPC (16:0)	496.33	$[M+H]^+$
2	LPC (18:1)	522.33	$[M+H]^+$
3	SM (16:0)	703.56	$[M+H]^+$
4	SM (16:0)	725.55	$[M+Na]^+$
5	SM (22:0)	787.56	$[M+H]^+$
6	SM (22:0)	809.55	$[M+Na]^+$
7	SM (23:0)	801.56	$[M+H]^+$
8	SM (23:0)	823.55	$[M+Na]^+$
9	SM (24:1)	813.56	$[M+H]^+$
10	SM (24:1)	835.55	$[M+Na]^+$
11	SM (24:0)	815.56	$[M+H]^+$
12	SM (24:0)	837.55	$[M+Na]^+$
13	PC (32:0)	734.56	$[M+H]^+$
14	PC (32:0)	756.55	$[M+Na]^+$
15	PC (34:2)	758.56	$[M+H]^+$
16	PC (34:2)	780.55	$[M+Na]^+$
17	PC (34:1)	760.56	$[M+H]^+$
18	PC (34:1)	782.55	$[M+Na]^+$
19	PC (36:2)	786.56	$[M+H]^+$
20	PC (36:2)	808.55	$[M+Na]^+$

2-2. Negative mode

No.	Lipid species	Detected *m/z*	Adduct ion
1	LPA (18:1)	435.25	$[M-H]^-$
2	LPA (18:0)	437.26	$[M-H]^-$
3	LPE (16:1)	450.26	$[M-H]^-$
4	LPE (22:1)	534.25	$[M-H]^-$
5	LPS (18:1)	522.27	$[M-H]^-$
6	LPS (22:5)	570.35	$[M-H]^-$
7	PA (36:1)	701.51	$[M-H]^-$
8	PE (O-36:5)	722.59	$[M-H]^-$
9	PE (36:2)	742.53	$[M-H]^-$
10	PE (O-38:5)	750.59	$[M-H]^-$
11	PE (38:4)	766.59	$[M-H]^-$
12	PS (34:1)	760.54	$[M-H]^-$
13	PS (36:1)	788.54	$[M-H]^-$
14	PS (38:4)	756.55	$[M-H]^-$
15	PS (40:2)	758.56	$[M-H]^-$
16	PI (34:1)	780.55	$[M-H]^-$
17	PI (36:4)	760.56	$[M-H]^-$
18	PI (38:6)	782.55	$[M-H]^-$
19	PI (38:4)	786.56	$[M-H]^-$
20	PI (42:6)	808.55	$[M-H]^-$

The urinary lipid species were extracted by the acidified B&D method and were analyzed in both positive and negative mode using 9-AA as the matrix. Finally, 11 lipid species, including 2 LPC, 5 SM, and 4 PC, were identified in the positive mode, while 20 lipid species, including 2 LPA, 2 LPE, 2 LPS, 1 PA, 4 PE, 4 PS, and 5 PI, were detected in the negative mode. The serial numbers of the lipids listed in this table correspond to the numbers in the peaks of the spectra shown in Fig. 7.

extraction and analysis system was validated using the endogenous urinary lipids. The detected urinary lipid species have been suggested to bear a relationship with a variety of human diseases, infections, and health risks. Therefore, our urinary screening method may become an effective and convenient tool for clinical laboratory examinations.

4. Conclusions

The MALDI-TOF/MS analysis for the study of lipids has proven to be effective and has become popular owing to several advantages of this technique. Moreover, as both lipids and the MALDI matrix are readily soluble in organic solvents, all manipulations can be performed in a single organic phase. This results in extremely homogeneous co-crystallization of the matrix and analytes, yielding excellent reproducibility compared with methods that require solvent mixtures with polar components. Lipid concentrations less than a few picomoles are sufficient for the MS detection and analysis (Wang et al., 2015; Pati et al., 2016; Leopold et al., 2018).

In this study, the procedures for the MALDI-TOF/MS analysis of urine samples were compared and optimized from the clinical perspective. An optimal MALDI matrix and lipid extraction method were identified using an internal STD mix of 13 lipids along with two ionization STDs spiked into human urine samples. 9-AA was a useful and effective matrix for all internal lipid STDs in both positive and negative modes, although it was necessary to confirm the proportioned concentration from the content balance between the extracted lipids and the matrix. The highest recoveries were obtained with the acidified B&D method, and reproducibility following acidified B&D extraction was excellent in both positive and negative ionization modes. The analysis of lipids in small volumes of urine by MALDI-TOF/MS was performed with the 9-AA matrix at concentrations $\leq$ 5 mM, following the acidified B&D lipid extraction. The combined analytical procedures can be used for rapid and comprehensive lipid screening of small quantities of PLs and LPLs in clinical samples.

Authors contribution

LX, KN, and TI contributed to the design, data acquisition, analysis, interpretation of results, and wrote the manuscript. TI, TG, SA, and OO contributed to recruiting clinical subjects, sample collection, and confirmation of clinical information. KS contributed to data investigation and analysis. OO and TI contributed to the overall management of the study, data investigation, data interpretation, and wrote the manuscript. All authors contributed to drafting the manuscript, and all authors have approved the final version of the manuscript.

Funding

This study was supported by Grants-in-Aid for Scientific Research (15K10587 and 18H02936) from the Japan Society for the Promotion of Science and by the Research Grant for Collaboration Research Projects from the Shimadzu Corporation.

Acknowledgements

The authors appreciate the kind cooperation of Shimadzu Corporation Group. They thank the members of the laboratory in the Department of Urology in the Graduate School of Medicine at Kyoto University for their intellectual support. They also thank Ms. Junko Hirao, Yukari Maruhashi, and Yasuko Kosai for their excellent technical assistance and Ms. Haruka Nishihara and Shiho Kayama for clinical research coordination. The authors also thank the Editage service provided by Cactus Communications Inc. (http://www.cactus.co.jp) for editing a draft of this manuscript.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.chemphyslip.2019.104787.

References

Angelini, R., Vitale, R., Patil, V.A., Cocco, T., Ludwig, B., Greenberg, M.L., Corcelli, A., 2012. Lipidomics of intact mitochondria by MALDI-TOF/MS. J. Lipid Res. 53, 1417–1425. https://doi.org/10.1194/jlr.D026203.

Barber, M.N., Risis, S., Yang, C., Meikle, P.J., Staples, M., Febbraio, M.A., Bruce, C.R., 2012. Plasma lysophosphatidylcholine levels are reduced in obesity and type 2 diabetes. PLoS One 7, e41456. https://doi.org/10.1371/journal.pone.0041456.

Bi, H., Krausz, K.W., Manna, S.K., Li, F., Johnson, C.H., Gonzalez, F.J., 2013. Optimization of harvesting, extraction, and analytical protocols for UPLC-ESI-MS-based metabolomic analysis of adherent mammalian cancer cells. Anal. Bioanal. Chem. 405, 5279–5289. https://doi.org/10.1007/s00216-013-6927-9.

Bligh, E.G., Dyer, W.J., 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. 37, 911–917. https://doi.org/10.1139/o59-099.
Bresler, K., Pyttel, S., Paasch, U., Schiller, J., 2011. Parameters affecting the accuracy of the MALDI-TOF MS determination of the phosphatidylcholine/lysophosphatidylcholine (PC/LPC) ratio as potential marker of spermatozoa quality. Chem. Phys. Lipids 164, 696–702. https://doi.org/10.1016/j.chemphyslip.2011.07.006.
Byeon, S.K., Lee, J.Y., Moon, M.H., 2012. Optimized extraction of phospholipids and lysophospholipids for nanoflow liquid chromatography-electrospray ionization-tandem mass spectrometry. Analyst 137, 451–458. https://doi.org/10.1039/c1an15920h.
Campa, C.C., Hirsch, E., 2017. Rab11 and phosphoinositides: a synergy of signal transducers in the control of vesicular trafficking. Adv. Biol. Regul. 63, 132–139. https://doi.org/10.1016/j.jbior.2016.09.002.
Chagovets, V., Lísa, M., Holčapek, M., 2015. Effects of fatty acyl chain length, double-bond number and matrix on phosphatidylcholine responses in matrix-assisted laser desorption/ionization on an Orbitrap mass spectrometer. Rapid Commun. Mass Spectrom. 29, 2374–2384. https://doi.org/10.1002/rcm.7404.
Cheng, H., Sun, G., Yang, K., Gross, R.W., Han, X., 2010. Selective desorption/ionization of sulfatides by MALDI-MS facilitated using 9-aminoacridine as matrix. J. Lipid Res. 51, 1599–1609. https://doi.org/10.1194/jlr.D004077.
Cruz, M., Wang, M., Frisch-Daiello, J., Han, X., 2016. Improved butanol–methanol (BUME) method by replacing acetic acid for lipid extraction of biological samples. Lipids 51, 887–896. https://doi.org/10.1007/s11745-016-4164-7.
Duncan, M.W., Roder, H., Hunsucker, S.W., 2008. Quantitative matrix-assisted laser desorption/ionization mass spectrometry. Brief. Funct. Genomic. Proteomic. 7, 355–370. https://doi.org/10.1093/bfgp/eln041.
Edidin, M., 1997. Lipid microdomains in cell surface membranes. Curr. Opin. Struct. Biol. 7, 528–532. https://doi.org/10.1016/S0959-440X(97)80117-0.
Folch, J., Lees, M., Sloane Stanley, G.H., 1957. A simple method for the isolation and purification of total lipides from animal tissues. J. Biol. Chem. 226, 497–509.
Fuchs, B., Bischoff, A., Süss, R., Teuber, K., Schürenberg, M., Suckau, D., Schiller, J., 2009. Phosphatidylcholines and -ethanolamines can be easily mistaken in phospholipid mixtures: a negative ion MALDI-TOF MS study with 9-aminoacridine as matrix and egg yolk as selected example. Anal. Bioanal. Chem. 395, 2479–2487. https://doi.org/10.1007/s00216-009-3032-1.
Fuchs, B., Süß, R., Schiller, J., 2010. An update of MALDI-TOF mass spectrometry in lipid research. Prog. Lipid Res. 49, 450–475. https://doi.org/10.1016/j.plipres.2010.07.001.
Gellermann, G.P., Appel, T.R., Davies, P., Diekmann, S., 2006. Paired helical filaments contain small amounts of cholesterol, phosphatidylcholine and sphingolipids. Biol. Chem. 387, 1267–1274. https://doi.org/10.1515/BC.2006.157.
Graessler, J., Mehnert, C.S., Schulte, K.M., Bergmann, S., Strauss, S., Bornstein, T.D., Licinio, J., Wong, M.L., Birkenfeld, A.L., Bornstein, S.R., 2018. Urinary Lipidomics: evidence for multiple sources and sexual dimorphism in healthy individuals. Pharmacogenomics J. 18, 331–339. https://doi.org/10.1038/tpj.2017.24.
Hara, A., Radin, N.S., 1978. Lipid extraction of tissues with a low toxicity solvent. Anal. Biochem. 90, 420–426. https://doi.org/10.1016/0003-2697(78)90046-5.
Jensen, S.K., 2008. Improved Bligh and Dyer extraction procedure. Lipid Technol. 20, 280–281. https://doi.org/10.1002/lite.200800074.
Kim, H., Ahn, E., Moon, M.H., 2008. Profiling of human urinary phospholipids by nanoflow liquid chromatography/tandem mass spectrometry. Analyst 133, 1656. https://doi.org/10.1039/b804715d.
Kim, H., Min, H.K., Kong, G., Moon, M.H., 2009. Quantitative analysis of phosphatidylcholines and phosphatidylethanolamines in urine of patients with breast cancer by nanoflow liquid chromatography/tandem mass spectrometry. Anal. Bioanal. Chem. 393, 1649–1656. https://doi.org/10.1007/s00216-009-2621-3.
Kuchař, L., Asfaw, B., Poupětová, H., Honzíková, J., Tureček, F., Ledvinová, J., 2013. Direct tandem mass spectrometric profiling of sulfatides in dry urinary samples for screening of metachromatic leukodystrophy. Clin. Chim. Acta 425, 153–159. https://doi.org/10.1016/j.cca.2013.06.027.
Leopold, J., Popkova, Y., Engel, K.M., Schiller, J., 2018. Recent developments of useful MALDI matrices for the mass spectrometric characterization of lipids. Biomolecules 8. https://doi.org/10.3390/biom8040173.
Löfgren, L., Ståhlman, M., Forsberg, G.-B., Saarinen, S., Nilsson, R., Hansson, G.I., 2012. The BUME method: a novel automated chloroform-free 96-well total lipid extraction method for blood plasma. J. Lipid Res. 53, 1690–1700. https://doi.org/10.1194/jlr.D023036.
Matyash, V., Liebisch, G., Kurzchalia, T.V., Shevchenko, A., Schwudke, D., 2008. Lipid extraction by methyl- tert -butyl ether for high-throughput lipidomics. J. Lipid Res. 49, 1137–1146. https://doi.org/10.1194/jlr.D700041-JLR200.
Min, H.K., Lim, S., Chung, B.C., Moon, M.H., 2011. Shotgun lipidomics for candidate biomarkers of urinary phospholipids in prostate cancer. Anal. Bioanal. Chem. 399, 823–830. https://doi.org/10.1007/s00216-010-4290-7.
Mulder, C., Wahlund, L.O., Teerlink, T., Blomberg, M., Veerhuis, R., Van Kamp, G.J., Scheltens, P., Scheffer, P.G., 2003. Decreased lysophosphatidylcholine /phosphatidylcholine ratio in cerebrospinal fluid in Alzheimer's disease. J. Neural Transm. 110, 949–955. https://doi.org/10.1007/s00702-003-0007-9.
Nagata, S., Suzuki, J., Segawa, K., Fujii, T., 2016. Exposure of phosphatidylserine on the cell surface. Cell Death Differ. 23, 952–961. https://doi.org/10.1038/cdd.2016.7.
Nishihara, M., Koga, Y., 1987. Extraction and composition of polar lipids from the archaebacterium, *Methanobacterium thermoautotrophicum*: effective extraction of tetraether lipids by an acidified solvent. J. Biochem. 101, 997–1005. https://www.jstage.jst.go.jp/article/biochemistry1922/101/4/101_4_997/_pdf/-char/en.
Orešič, M., Hyötyläinen, T., Herukka, S.K., Sysi-Aho, M., Mattila, I., Seppänan-Laakso, T., Julkunen, V., Gopalacharyulu, P.V., Hallikainen, M., Koikkalainen, J., Kivipelto, M., Helisalmi, S., Lötjönen, J., Soininen, H., 2011. Metabolome in progression to Alzheimer's disease. Transl. Psychiatry 13 (1), e57. https://doi.org/10.1038/tp.2011.55.
Pati, S., Nie, B., Arnold, R.D., Cummings, B.S., 2016. Extraction, chromatographic and mass spectrometric methods for lipid analysis. Biomed. Chromatogr. 30, 695–709. https://doi.org/10.1002/bmc.3683.
Pietruszko, R., Gray, G.M., 1962. The products of mild alkaline and mild acid hydrolysis of plasmalogens. Biochim. Biophys. Acta 56, 232–239. https://doi.org/10.1016/0006-3002(62)90560-7.
Pyttel, S., Zschörnig, K., Nimptsch, A., Paasch, U., Schiller, J., 2012. Enhanced lysophosphatidylcholine and sphingomyelin contents are characteristic of spermatozoa from obese men - A MALDI mass spectrometric study. Chem. Phys. Lipids 165, 861–865. https://doi.org/10.1016/j.chemphyslip.2012.11.001.
Reis, A., Rudnitskaya, A., Blackburn, G.J., Fauzi, N.M., Pitt, A.R., Spickett, C.M., 2013. A comparison of five lipid extraction solvent systems for lipidomic studies of human LDL. J. Lipid Res. 54, 1812–1824. https://doi.org/10.1194/jlr.M034330.
Retra, K., Bleijerveld, O.B., Van Gestel, R.A., Tielens, A.G.M., Van Hellemond, J.J., Brouwers, J.F., 2008. A simple and universal method for the separation and identification of phospholipid molecular species. Rapid Commun. Mass Spectrom. 22, 1853–1862. https://doi.org/10.1002/rcm.3562.
Rockwell, H.E., Gao, F., Chen, E.Y., McDaniel, J., Sarangarajan, R., Narain, N.R., Kiebish, M.A., 2016. Dynamic assessment of functional lipidomic analysis in human urine. Lipids. 51, 875–886. https://doi.org/10.1007/s11745-016-4142-0.
Röst, H.L., Sachsenberg, T., Aiche, S., Bielow, C., Weisser, H., Aicheler, F., Andreotti, S., Ehrlich, H.C., Gutenbrunner, P., Kenar, E., Liang, X., Nahnsen, S., Nilse, L., Pfeuffer, J., Rosenberger, G., Rurik, M., Schmitt, U., Veit, J., Walzer, M., Wojnar, D., Wolski, W.E., Schilling, O., Choudhary, J.S., Malmström, L., Aebersold, R., Reinert, K., Kohlbacher, O., 2016. OpenMS: a flexible open-source software platform for mass spectrometry data analysis. Nat. Methods 13, 741–748. https://doi.org/10.1038/nmeth.3959.
Schiller, J., Süß, R., Arnhold, J., Fuchs, B., Leßig, J., Müller, M., Petković, M., Spalteholz, H., Zschörnig, O., Arnold, K., 2004. Matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry in lipid and phospholipid research. Prog. Lipid Res. 43, 449–488. https://doi.org/10.1016/j.plipres.2004.08.001.
Schiller, J., Suss, R., Fuchs, B., Muller, M., Zschornig, O., Arnold, K., 2007. MALDI-TOF MS in lipidomics. Front. Biosci. 12, 2568–2579. https://doi.org/10.2741/2255.
Serna, J., García-Seisdedos, D., Alcázar, A., Lasunción, M.Á., Busto, R., Pastor, Ó., 2015. Quantitative lipidomic analysis of plasma and plasma lipoproteins using MALDI-TOF mass spectrometry. Chem. Phys. Lipids 189, 7–18. https://doi.org/10.1016/j.chemphyslip.2015.05.005.
Solouki, T., White, F.M., Marto, J.A., Guan, S., Marshall, A.G., 1995. Attomole biomolecule mass analysis by matrix-assisted laser desorption/ionization fourier transform ion cyclotron resonance. Anal. Chem. 67, 4139–4144. https://doi.org/10.1021/ac00118a017.
Spacil, Z., Kumar, A.B., Liao, H.C., Auray-Blais, C., Stark, S., Suhr, T.R., Ronald Scott, C., Turecek, F., Gelb, M.H., 2016. Sulfatide analysis by mass spectrometry for screening of metachromatic leukodystrophy in dried blood and urine samples. Clin. Chem. 62, 279–286. https://doi.org/10.1373/clinchem.2015.245159.
Stübiger, G., Aldover-Macasaet, E., Bicker, W., Sobal, G., Willfort-Ehringer, A., Pock, K., Bochkov, V., Widhalm, K., Belgacem, O., 2012. Targeted profiling of atherogenic phospholipids in human plasma and lipoproteins of hyperlipidemic patients using MALDI-QIT-TOF-MS/MS. Atherosclerosis 224, 177–186. https://doi.org/10.1016/j.atherosclerosis.2012.06.010.
Sun, G., Yang, K., Zhao, Z., Guan, S., Han, X., Gross, R.W., 2008. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometric analysis of cellular glycerophospholipids enabled by multiplexed solvent dependent analyte-matrix interactions. Anal. Chem. 80, 7576–7585. https://doi.org/10.1021/ac801200w.
Szájli, E., Fehér, T., Medzihradszky, K.F., 2008. Investigating the quantitative nature of MALDI-TOF MS. Mol. Cell Proteomics 7, 2410–2418. https://doi.org/10.1074/mcp.M800108-MCP200.
Vanni, S., 2017. Intracellular lipid droplets: from structure to function. Lipid Insights 10, 14–16. https://doi.org/10.1177/1178635317745518.
Wang, C., Wang, M., Han, X., 2015. Applications of mass spectrometry for cellular lipid analysis. Mol. Biosyst. 11, 698–713. https://doi.org/10.1039/c4mb00586d.
Wang, H., Wong, C.H., Chin, A., Taguchi, A., Taylor, A., Hanash, S., Sekiya, S., Takahashi, H., Murase, M., Kajihara, S., Iwamoto, S., Tanaka, K., 2011. Integrated mass spectrometry-based analysis of plasma glycoproteins and their glycan modifications. Nat. Protoc. 6, 253–269. https://doi.org/10.1038/nprot.2010.176.
Wang, P., Giese, R.W., 2017. Recommendations for quantitative analysis of small molecules by matrix-assisted laser desorption ionization mass spectrometry. J. Chromatogr. A 1486, 35–41. https://doi.org/10.1016/j.chroma.2017.01.040.
Zhao, Z., Xiao, Y., Elson, P., Tan, H., Plummer, S.J., Berk, M., Aung, P.P., Lavery, I.C., Achkar, J.P., Li, L., Casey, G., Xu, Y., 2007. Plasma lysophosphatidylcholine levels: potential biomarkers for colorectal cancer. J. Clin. Oncol. 25, 2696–2701. https://doi.org/10.1200/JCO.2006.08.5571.
Zhou, Y., Wong, C.O., Cho, K.J., Van Der Hoeven, D., Liang, H., Thakur, D.P., Luo, J., Babic, M., Zinsmaier, K.E., Zhu, M.X., Hu, H., Venkatachalam, K., Hancock, J.F., 2015. Membrane potential modulates plasma membrane phospholipid dynamics and K-Ras signaling. Science. 349, 873–876. https://doi.org/10.1126/science.aaa5619.