Doctoral Dissertation

# Simultaneous profiling of polar lipids by supercritical fluid chromatography/tandem mass spectrometry with derivatization for lipidomics

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Index

# Abbreviation

| | | | |
|---|---|---|---|
| ABPR | Atmospheric pressure | MSTFA | *N*-methy-*N*-(trimethylsilyl) trifluoroacetamide |
| AD | Degree Celsius | *m/z* | Mass to charge ratio |
| APCI | Electronvolt | NMR | Nuclear magnetic resonance |
| BSA | *N*, *O*-bis(trimethylsilyl) acetamide | ODS | Octadecylsilylated |
| BSTFA | *N*, *O*-bis(trimethylsilyl) trifluoroacetamide | PD | Parkinson's diseases |
| C | Carbon | Q | Quadrupole |
| CE | Collision energy | QqQ | Triple quadrupole |
| CID | Collision-induced dissociation | $R^2$ | Correlation |
| CN | Cyano | RF | Radio frequency |
| Cn | Total chain length | RP | Reversed-phase |
| $CO_2$ | Carbon dioxide | RSD | Relative standard deviation |
| CV | Cone voltage | RT | Retention time |
| EDTA | Ethylenediaminetetraacetic acid | $SCCO_2$ | Supercritical carbon dioxide |
| ESI | Electrospray ionization | SF | Supercritical fluid |
| GC | Gas chromatography | SFC | Supercritical fluid chromatography |
| HETP | Height equivalent to a theoretical plate | *Sn* | Stereospecific numbering |
| HPLC | High-performance liquid chromatography | S/N | Signal-to-noise ratios |
| ID | Internal diameter | TLC | Thin-layer chromatography |
| IS | Internal standard | TMCS | Trimethylchlorosilane |
| LOD | Limit of detection | TMS | Trimethylsilyl |
| LOQ | Limit of quantification | TMSD | Trimethylsilyldiazomethane |
| MALDI | Matrix assisted laser desorption ionization | TMSI | *N*-trimethylsilylimidazole |
| MRM | Multiple reaction monitoring | UHPLC | Ultra-high performance liquid chromatography |
| MS | Mass spectrometry | Un | Total degree of unsaturation |
| MS/MS | Tandem mass spectrometry | | |

## Units

| | | | | | |
|---|---|---|---|---|---|
| atm | Atmospheric pressure | MPa | Megapascal | min | Minute(s) |
| ºC | Degree Celsius | µL | Microliter | Mol | Mole |
| Da | Dalton | µg | Microgram | % | Percentage |
| eV | Electronvolt | µm | Micrometer | S | Second(s) |
| fmol | Femtomole | mL | Milliliter | × g | Times gravity |
| Hz | Hertz | mg | Milligram | V | Volt |
| h | hour(s) | mm | Millimeter | v/v | Volume per volume |
| L | Liter | | | | |

## Symbols for lipids

| | | | |
|---|---|---|---|
| Cer | Ceramide | PG | Phosphatidylglycerol |
| Cer1P | Ceramide-1-phosphate | PI | Phosphatidylinositol |
| FA | Fatty acid | PL | Phospholipid |
| LPA | Lysophosphatidic acid | PS | Phosphatidylserine |
| LPC | Lysophosphatidylcholine | PtdIns(3,4,5)$P_3$ | Phosphatidylinositol(3,4,5)-trisphosphate |
| LPE | Lysophosphatidylethanolamine | n-3 PUFA | Omega-3 polyunsaturated fatty acid |
| LPG | Lysophosphatidylglycerol | Sa | Sphinganine |
| LPI | Lysophosphatidylinositol | Sa1P | Sphinganine-1-phosphate |
| LPL | Lysophospholipid | SL | Sphingolipid |
| LPS | Lysophosphatidylserine | SM | Sphingomyelin |
| PA | Phosphatidic acid | So | Sphingosine |
| PC | Phosphatidylcholine | So1P | Sphingosine-1-phosphate |
| PE | Phosphatidylethanolamine | TAG | Triacylglycerol |

# Chapter 1
# General introduction

## 1.1 Lipidomics

### 1.1.1 Lipids

There are several ways to define lipids. At first, lipids are fatty acids (FAs) and their derivatives, and substances related biosynthetically or functionally to these compounds. Secondly, lipids also can be divided into two groups, “simple” and “complex” groups. Simple lipids are those that on hydrolysis yield at most two types of primary product, and complex lipids yield three or more primary hydrolysis products. Alternatively, the terms "neutral" and "polar" lipids are also used to define these groups. Thirdly, lipids are defined as hydrophobic or amphipathic small molecules that originate by carbanion-based condensations of thioesters (FAs, polyketides, etc.) and/or by carbocation-based condensations of isoprene units (prenols, sterols, etc.). Moreover, lipids are classified into eight categories, such as FAs, glycerolipids, phospholipids (PLs), sphingolipids (SLs), sterol lipids, prenol lipids, saccharolipids, and polyketides, containing distinct classes and subclasses of molecules. Random permutation of FAs with various backbone positions and combination with different head groups would theoretically produce thousands of lipid species. However, the nonrandom distribution in biological systems generates a smaller number of species than is theoretically possible [1,2].

Lipids play various roles in living organisms. They are cell membrane components that provide structural integrity to cells and organelles, and create an optimal physiological and chemical environment for protein interactions and functions [3]. They also serve as an energy reservoir and precursors for many messenger molecules [4]. The crucial role of lipids in cell signaling and tissue physiology is demonstrated by the large number of diseases and neurological disorders in which lipid metabolism is altered. Lipid metabolism is importance for the nervous system, as this organ has the second highest concentration of lipids, exceeded only by adipose tissue. In the field of lipid research, a large number of experiments using

genetic, cell biological and biochemical approaches in model organisms have advanced our knowledge of lipid biosynthesis and function [5,6]. Many of these studies have included direct measurement of lipid molecules. Furthermore, the characterization of complicated enzymatic machinery that acts on lipids as well as effectors of lipids has been studied [7,8].

### 1.1.2 Lipidomics and its platform

Remarkable advances in biotechnology have forged a new era of research in the field of systems biology that aims to decipher relationships between different parts of a biological system such as a metabolic pathway, cell, and tissue with the understanding the system's behavior as a whole [4]. The comprehensive investigations of living organisms at the molecular system level can be classified into different fields: genomics, transcriptomics, proteomics, and metabolomics, known as the "omics cascade" [9,10]. The integrative analysis of an organism's response to a perturbation on the "omics" levels will lead to a more complete understanding of the biochemical and biological mechanisms in complex systems.

Lipidomics is a critical part of metabolomics among the "omics cascade", and aims to study all the lipids within living organisms through a systems-level analysis of lipid species, their abundance, biological activities, and subcellular localization [11]. Lipidomics involves studies based on analytical techniques to obtain a lipid profile containing information on the composition and abundance of individual lipids as well as to understand the functions of lipids in a biological sample [12-14]. Recently, lipidomics research has captured increased attention due to the recognized roles of several lipids and the correlations between lipids and numerous human diseases such as diabetes, obesity, and atherosclerosis. Investigating lipid biochemistry using a lipidomics approach will not only provide insights into the specific roles of lipid molecular species in health and disease, but will also assist in identifying potential biomarkers for establishing preventive or therapeutic approaches for human health. However, lipidomics has been limited in its development by both the unclear definition of complex lipid

species and insufficient analytical technology.

Technological advances in mass spectrometry (MS) and chromatography have produced several lipidomic approaches capable of characterizing lipid species by direct analysis of total lipid extracts [15]. The development of soft ionization techniques such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and matrix assisted laser desorption ionization (MALDI) have significantly expanded the range of lipids that can be analyzed qualitatively and quantitatively by MS [16,17]. Among these ionization techniques, ESI is most widely used for lipid analysis. For instance, ESI-based shotgun lipidomics, the direct mass spectrometric analysis of cellular lipidomes from crude extracts, was successfully utilized to investigate lipid perturbation [18-20]. ESI allows the analysis of large, nonvolatile molecules directly from the liquid phase. It rarely disrupts the chemical nature of the analyte prior to mass analysis. The sample is introduced through a capillary tube into the ion source of the mass spectrometer in a water-rich solvent at atmospheric pressure. A high voltage (2–5 kV) is applied to the capillary relative to the entrance of the mass spectrometer, which creates an electric field gradient along which the charged droplets travel. As they travel, the solvent rapidly evaporates in a curtain gas of nitrogen and the density of the charges increases to the point of coulombic repulsion. At this point, large droplets divide into smaller droplets and eventually into individual charged molecules, which enter the mass spectrometer.

In addition, the separation by chromatography is essential for a comprehensive analysis of diverse lipids due to their high complexity. High-performance liquid chromatography (HPLC) coupled to ESI/MS or ESI/MS/MS is sensitive and critical techniques to analyze lipids, especially for minor lipid species [21-23]. Other chromatographic techniques such as thin-layer chromatography (TLC) [24], gas chromatography (GC) [25,26], ultra-high performance liquid chromatography (UHPLC) [27,28], and supercritical fluid chromatography (SFC) [29-31] are also used widely for the analysis of lipids. The evolution of “omics cascade” from genomics to lipidomics and the platform of lipidomics were described in Figure 1.1.

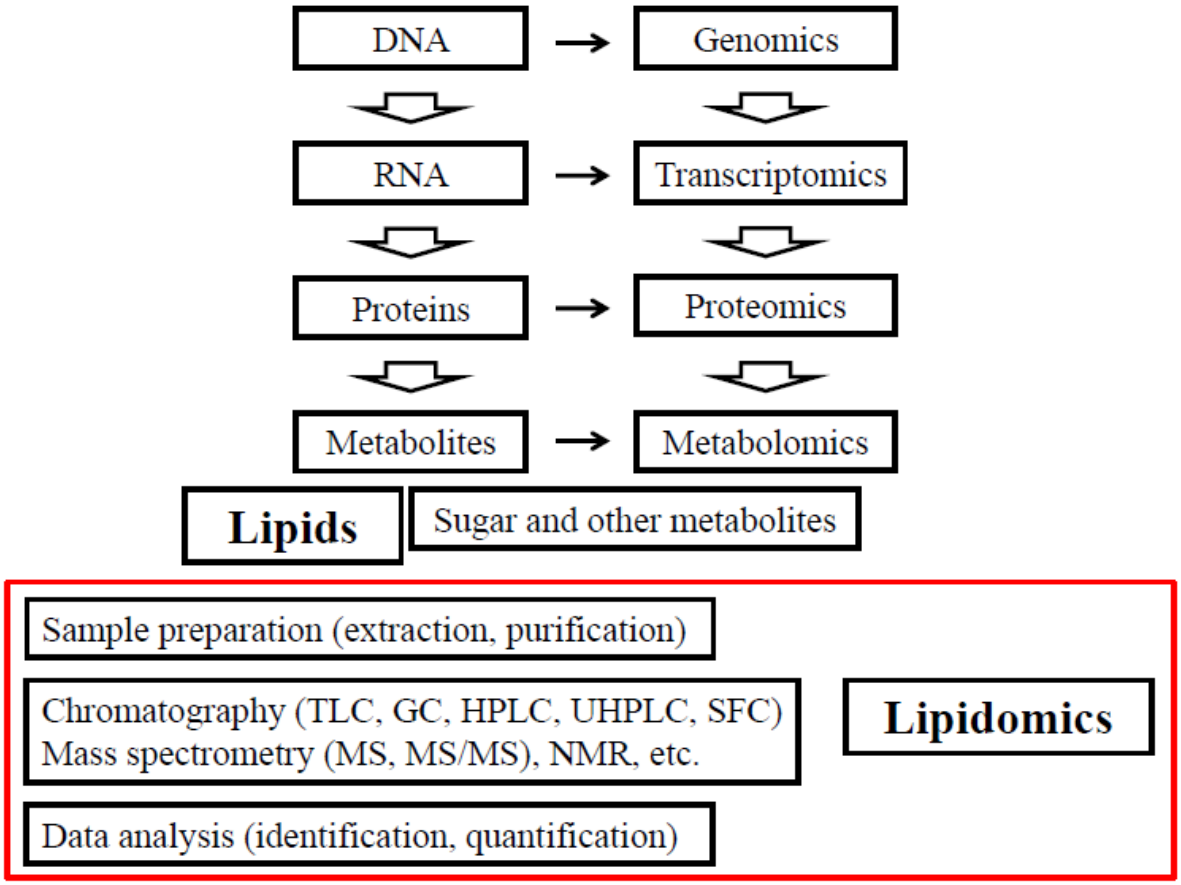


**Figure 1.1** The evolution of "omics cascade" from genomics to lipidomics and the platform of lipidomics. Genomics: mapping the entire genome (DNA). Transcriptomics: examining the expression level of mRNAs, non-coding RNAs, and small RNAs to determine the transcriptional structure of genes. Proteomics: identifying, sequencing and characterizing the functional protein network. Metabolomics: the comprehensive analysis of the whole metabolome under a given set of conditions. Lipidomics: the systems-level analysis and characterization of total lipids and their interacting moieties.

### 1.1.3 Aberrant lipid metabolism in human diseases

Lipid levels are tightly regulated, spatially and temporally, in the various parts of the human body, and it proposes that deregulation of this network contributes to the onset of pathology. This idea is supported by a wide variety of human diseases that are associated with aberrant lipid metabolism [4-6, 32]. Many studies have provided valuable insights into the

relationship between metabolic changes in lipids and human diseases/disorders. For example, the aberrant lipid metabolism was reported as a critical contributor to the etiology of obesity and related metabolic diseases such as diabetes, fatty liver disease and atherosclerosis [33-35]. Metabolic abnormalities including dyslipidemia and glucose intolerance were also occurred in obesity [36-38]. In the case of atherosclerosis that is induced by a chronic inflammatory response, the oxidative and enzymatic modifications of low density lipoprotein lead to the release of inflammatory lipids that induce the immune response [39]. Lipid metabolism and its complex output of lipid species play a key role in the development of metabolic syndrome. Many studies have shown that modified PLs lead to reactive species which are able to promote atherogenesis by interacting with proteins, cells, and the immune system [40]. Furthermore, in cell signaling events, various lipids such as 3-phosphoinositides work to control cellular growth and death so that lipid signaling is in interests to develop novel treatments for cancer and respiratory disease [41,42]. Lipid metabolism is also critical to the central nervous system [43]. The crucial role of lipids in cell signaling and tissue physiology is demonstrated by many studies of neurological disorders such as bipolar disorders and schizophrenia [44,45], and neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (PD) that involve deregulated lipid metabolism [46-48]. For example, in a toxin-induced mouse model of PD, a neuroprotective role for omega-3 polyunsaturated fatty acids (n-3 PUFAs) was found [49].

Lipidomics studies of dysfunctional lipid metabolism can be performed by using a variety of cell culture models, mouse models and tissue samples from humans. Such studies have provided valuable insights into the metabolic consequences of human diseases that have the aberrant lipid metabolism [50]. The altered lipid profiles in biological species have been screened to identify biomarkers [51-53]. Lipidomics is useful to obtain a molecular signature to a certain metabolic pathway or a disease condition. Furthermore, lipidomics together with RNA silencing may provide powerful tools to elucidate the specific roles of lipid intermediates in cell signaling and open new opportunities for drug development [2,54].

## 1.2 Polar lipid profiling

### 1.2.1 Polar lipids

Lipids are normally defined as hydrophobic or amphipathic small molecules that originate by thioesters and/or isoprene units. Alternatively, amphipathic lipids can be called as polar lipids that have relatively high polarity comparing to non-polar lipids. Polar lipids consist of one hydrophilic head group and one or two hydrophobic fatty acyl tails. In particular, PLs, lysophospholipids (LPLs), and SLs are classified as polar lipids. The PL structures have variations in substitution at each of the three carbon atoms in a glycerol. The stereospecific numbering (*sn*)-3 carbon participates in a phosphodiester linkage to various head groups result in different classes: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidic acid (PA), phosphatidylglycerol (PG), and phosphatidylinositol (PI). The *sn*-1 and *sn*-2 carbons participate in a carboxylic acid ester (acyl) linkage to a FA substituent, and molecular diversity arises from FA substituents with various side chains (called 'molecular species'), e.g., arachidonate, linoleate, oleate, etc [1,55] (Figure 1.2A). LPLs have one FA moiety at the *sn*-1 or *sn*-2 position of the glycerol backbone. Various polar head groups in LPLs have resulted in different classes: lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylserine (LPS), lysophosphatidic acid (LPA), lysophosphatidylglycerol (LPG), and lysophosphatidylinositol (LPI) (Figure 1.2B). PLs and LPLs have different types of head groups and combinations of FAs that vary in chain length and degree of unsaturation [56]. SLs also comprise complex molecules by the variation in the head group attached to the primary hydroxyl group, N-acyl group, and sphingoid-base backbone would change the nature and characteristics of the SL [57]. In the biosynthesis of SL, the major lipids are sphingomyelin (SM), ceramide (Cer), sphingosine (So), sphinganine (Sa), and phospho-SLs including ceramide-1-phosphate (Cer1P), sphingosine-1-phosphate (So1P), and sphinganine-1-phosphate (Sa1P) [58] (Figure 1.2C).

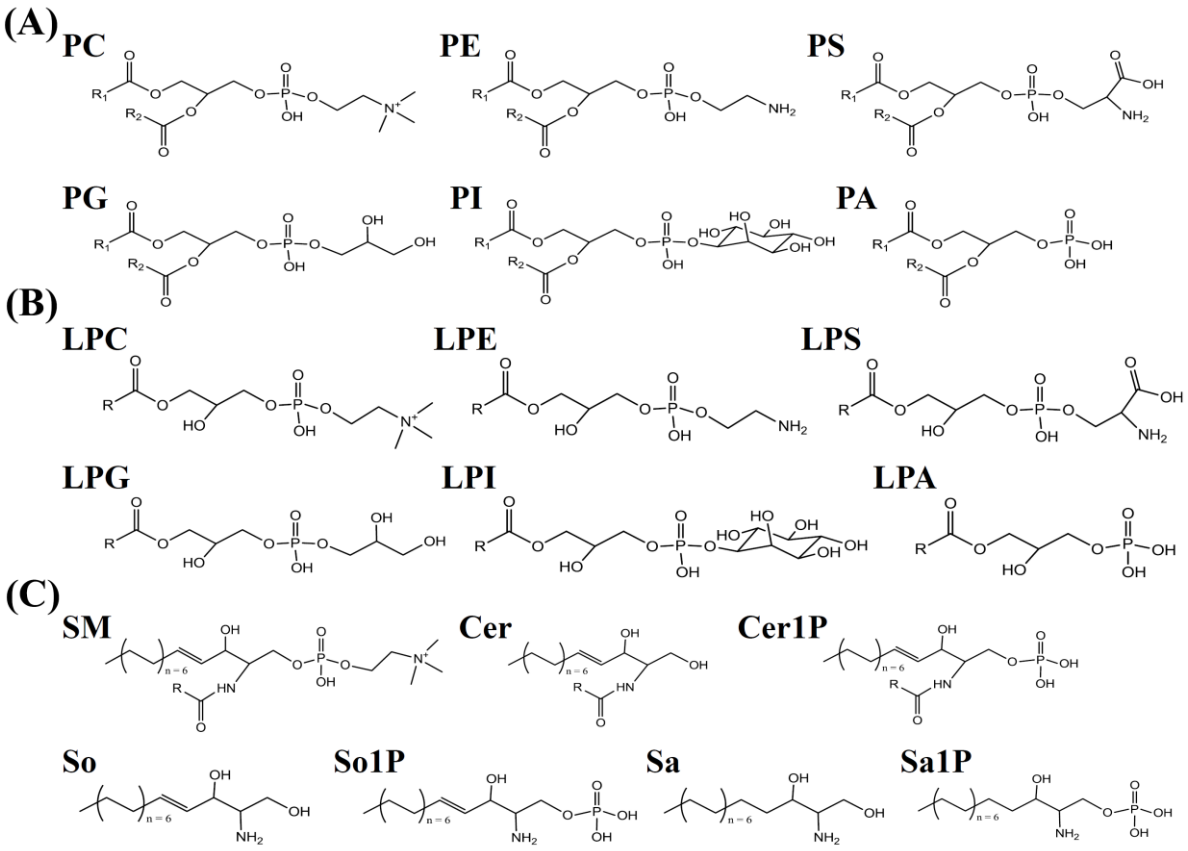


**Figure 1.2** The molecular structure of polar lipids: phospholipids (A), lysophospholipids (B), and sphingolipids (C).

These complex polar lipids have critical functions in the living organism. Polar lipids formed the matrix of cellular membranes due to its amphipathic property by a hydrophobic and a hydrophilic portion. The propensity of the hydrophobic moieties to self-associate and the tendency of the hydrophilic moieties to interact with aqueous environments and with each other are the physical basis of the spontaneous formation of membranes. In the membrane, polar lipids provide not only the barrier functions to maintain the homeostasis of cells as the components of cell membranes [59-61] but also the potential for budding, tabulation, fission and fusion, and the essential characteristics for cell division, biological reproduction, and intracellular membrane trafficking. Furthermore, polar lipids function as first and second messengers in signal transduction and molecular recognition processes. In particular, PLs and LPLs function as the mediation of signal transduction and ligands of receptors [62,63]. SLs also

serve not only as the membrane components but also as bioactive compounds having crucial biological functions. These various polar lipids such as PLs, LPLs, and SLs are studied widely to understand their biological functions. However, due to the high complexity of molecules, it remains be a challenging work to determine each polar lipid species.

### 1.2.2 Altered profile of polar lipids in human diseases

Polar lipid compositions are regulated in vivo by a combination of nutrition and phenotypic expression, and these compositions are characteristic of different cell types and organs [50]. Different membranes and organelles within a cell, as well as the inner and outer leaflets of the membrane bilayer, have very different lipid compositions. The crucial role of various polar lipids is demonstrated by a large number of genetic studies and many human diseases that involve the disruption of lipid metabolic enzymes and pathways [64,65]. In particular, advances in analytical methods have allowed us to find the altered profile of polar lipids in several human diseases. Polar lipids have recently shown to be involved in many physiological and pathological processes such as inflammation, reproduction, angiogenesis, tumorogenesis, atherosclerosis and nervous system regulation [56]. Levels of SLs are altered in various forms of cancers. Elevated levels of Cer are associated with cell death, whereas So1P promotes cell growth and migration. Cer can be generated by a number of pathways and both degradation of SM, as well as de novo synthesis of Cer, have been implicated in apoptosis [66,67]. Cancer cells perform their malicious activities through own membranes, and the alterations of PC and PE were found in the membrane of cancer cell [68]. The change of LPL levels was also found in cancer cells [69,70], and PS and PI were detected as the biomarkers for a prostate cancer [71]. In the previous study, a decrease of sulfatides and increased levels of Cer were observed in Alzheimer's diseases (AD) [72]. Furthermore, Changes in membrane PLs occur very early in AD pathogenesis and may provide the molecular basis for synaptic loss in AD [73-75]. It was also found that the FA profile of erythrocyte membrane PLs and activity of desaturase

enzymes are strongly linked to the incidence of diabetes [76]. The compositions of Cer were changed in diabetes [77]. In addition, the altered compositions of PLs and PUFAs were found in atherosclerosis [78], inflammatory disease [79], cardiovascular disease [80,81], and insulin resistance [82]. In conclusion, the altered profiles of PLs, LPLs, and SLs were found in several human diseases, and it demonstrated that there is the correlation between these lipids and certain diseases. Therefore, in the study of human diseases, polar lipid profiling is useful to screen the altered polar lipids not only for identifying biomarkers but also for understanding the aberrant metabolism of polar lipids in diseases. Additionally, an effective analytical system is required for the detail characterization of various polar lipids.

### 1.2.3 Analytical methods for polar lipids

In order to develop an effective analytical method for polar lipid profiling, several chromatographic methods such as high HPLC [83-85], UHPLC [86,87], and SFC [31,88] are widely used for the separation of polar lipids. Furthermore, high-sensitive and high-selective MS [89,90] is effective for the identification and quantification of various lipid species. Detailed profiling of PLs has been performed by reversed-phase (RP) HPLC [91]. The global profiling of 62 PLs and 50 LPLs in human plasma was performed by nanoflow LC/MS/MS [92]. Ag-adduction has also been used for the regiochemical study of PLs [60]. In addition, LC/MS/MS has emerged as a powerful tool for the analysis of SLs [93,94]. The analysis of phospho-SLs such as Cer1P and So1P was also reported [95]. Especially, the derivatization by naphthalene-2,3-dicarboxaldehyde improved So1P analysis [96]. Many methods have been developed for the profiling of various polar lipids such as PLs, LPLs, and SLs.

However, these methods are not well suited for the simultaneous analysis of PLs due to the severe peak tailing of acidic PLs such as PA and PS. The peak shape must be advanced because the peak tailing lowers the detection sensitivity of compound. If the peak shape is too broad due to the tailing, its peak height will be lowered. Thus, its intensity might be decreased

even with the same area. In the case that a low-abundance compound has peak tailing, its detection will be hindered by other noise peaks. Acidic PLs having peak tailing are normally low-abundance in biological samples. Finally, it is difficult to analyze acidic PLs having peak tailing in spite of their presence in biological samples. Therefore, the peak tailing must be advanced for the detail and comprehensive profiling of polar lipids. To achieve this goal, the main factor of peak tailing is needed to clarify. A previous paper reported that phosphate has high metal-affinity, and its absorption to the stainless steel of column causes the peak tailing [97]. Thus, the reason of peak tailing in PA that has phosphate was found. In the case of PS that has no phosphate, it also showed peak tailing. In order to clarify why PS also has peak tailing, a flow injection analysis that uses no column but only a syringe needle was performed. As a result, PS showed peak tailing even without column. Finally, it was proved that PS also has metal-affinity [98].

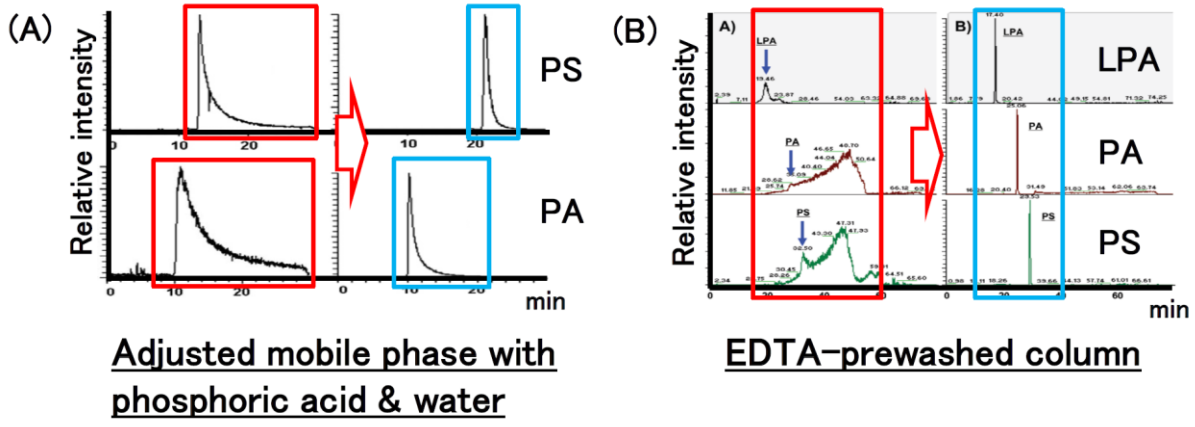


**Figure 1.3** Suppressed peak tailings of PS, PA, and LPA by two previous methods: Ogiso *et al.* (A) and Sato *et al.* (B).

In order to improve the peak shape of acidic PLs, two special methods have been developed to suppress the metal-affinity of compounds. Ogiso *et al.* [98] used a starting mobile phase containing a low concentration of phosphoric acid (5μmol/L) and a high concentration

of water (40%), and developed RP-LC conditions to suppress PA and PS peak tailing (Figure 1.3A). However, peak tailing for LPA could not be completely eliminated, and low-abundance species of PLs could not be analyzed. Moreover, the use of much phosphoric acid which is non-volatile can give harmful effects on the ESI probe, and this method is limited only to the analysis of PA and PS. In order to solve these limitations, Sato *et al.* [99] suggested that prewashing the analytical column by ethylenediaminetetraacetic acid (EDTA) in PL analysis has resulted in improved peak shape and sensitivity to PA, PS, LPA, and LPS (Figure 1.3B). Furthermore, quantitative analysis of low-abundance species of PLs in biological samples was possible. However, this method needs the use of non-metal system because the metals of not only column but also other parts such as syringe needle can cause the peak tailing. Moreover, the repeatability of system can be changed when the number of injection is increased and the condition of column washed by EDTA is changed. These several demerits still have hindered the practical use of above two methods so that it is required to develop more effective method to obtain the comprehensive and sensitive profiling of polar lipids with sharp peak shape.

Previous two methods have targeted to modify the chromatographic conditions such as the mobile phase and column to suppress the peak tailing. However, the modified conditions are not easy to control and optimize for high repeatability so that it remains to be challenging to develop a more effective method to improve the peak shape. To achieve this goal, I focused on modifying not the chromatographic conditions but the target compounds by derivatization. Derivatization was useful to block several functional groups in the head group of polar lipids so that it might be able to suppress the metal-affinity of compounds. The hydrophobicity of polar lipids was also increased by blocking the hydrophilic head group. In particular, SFC is effective for the analysis of hydrophobic compounds. Thus, in this study, SFC was used to analyze the derivatized polar lipids. For the high-sensitive and high-selective detection of various polar lipid species, MS/MS was applicable. Finally, SFC/MS/MS with derivatization was used to develop an effective analytical method for polar lipids.

### 1.3 SFC/MS/MS with derivatization

#### 1.3.1 Derivatization

Derivatization is a technique that transforms a target compound to a product compound called a derivative. It is carried out by specific chemical reactions between reactive functional groups in the target compounds and the corresponding reaction groups in the derivative reagent (Figure 1.4). This reaction changes the characteristics of derivate (e.g. deviating reactivity, solubility, boiling point, melting point, aggregate state, or chemical composition, etc.). Resulting new chemical properties is applicable for the quantification or separation of target compounds. However, derivatization for the purpose of analytical determination is only recommended when it is necessary to do so, because it is time-consuming and the error to quantitative determination can be caused by chemical manipulation. Therefore, it is necessary to take precautions and to include suitable control experiments. The reaction parameters such as derivative reagent amount, reaction time, temperature, catalyst, quenching solvent, and the working-up procedure are also required to be optimized and controlled to obtain a high and reproducible yield. Moreover, the stability of derivatized products should be evaluated.

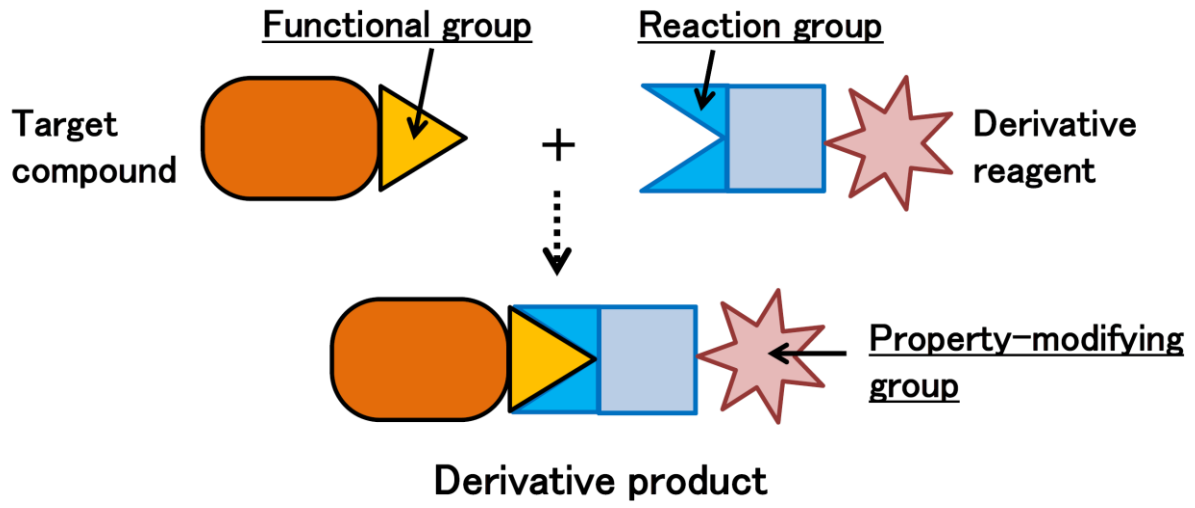


**Figure 1.4** Derivatization

**Table 1.1** The purposes of derivatization procedures integrated with LC/MS

| Steps | Purposes |
|---|---|
| Chromatographic separation | Adjust retention time on conventional RP column |
| | Advance peak shape/symmetry |
| | Increase resolution/chiral separation |
| MS detection | Improve selectivity by increasing molecular weight |
| | Enhance neubulization & ionization efficiency |
| | Facilitate structure elucidation |
| | Aid fragmentation for MRM |
| | Extend the linear dynamic range of calibration curve |

The integration of LC/MS with chemical derivatization is a relatively new and unique strategy that could enhance the capabilities of LC/MS based technologies [100]. Derivatization could be carried out in various analytical steps such as chromatographic separation and MS detection, and its purposes are listed in Table 1.1. In particular, derivatization is useful to improve peak shape. Compounds having both acidic and basic groups usually show a tailing or split peak in a chromatogram. The alternation of their chemical properties by derivatization will produce symmetry peaks [101], and it is useful to improve the analysis by LC/MS.

In the chemical derivatization, there are several kinds of methods such as acylation [102], alkylation [103], and esterification [104,105] (Table 1.2), and it is important to select the suitable derivatization according to the target functional group. In particular, polar lipids mainly have four functional groups such as hydroxyl (-OH), amine ($-NH_2$), carboxyl (-COOH), and phosphate ($-PO_4H_2$) groups in their structures, and various derivatizations can be applied for these groups. According to the kind of used derivatization method and reagent, the chemical reaction and required manipulation are different. Some derivatization is difficult to apply for polar lipid profiling due to its several demerits. For example, in the acylation of hydroxyl or amine group by using the acetyl chloride, hydrogen chloride (HCl) is released in the reaction. Then, the use of basic receptor is required to remove the excess HCl, and it is required to perform the additional extraction procedure. Furthermore, in the Fischer esterification, $H_2O$ is

released in the reaction so that the work-up procedure is required to remove $H_2O$ in the mixture. If the manipulation is complex, it will become time-consuming and handy-error rate will be increased. These can give unfavorable effects on the accuracy and practicality of polar lipid profiling. Therefore, in the selection of derivatization, the simplicity is a critical point to suppress the time-consuming and handy-error.

**Table 1.2** Derivatization methods

| Derivatizations | Reagents | Functional groups | Features |
|---|---|---|---|
| Acylation | Acid anhydrides<br>Acid halides<br>Reactive acyl derivatives | -OH, -SH<br>$-NH_2$, -NH-R<br>-NH-CO-R | Released acids (HCl)<br>(Use of basic acceptor & work-up procedure) |
| Alkylation<br>(e.g. methylation) | Alkyl halides<br>Diazoalkanes<br>(e.g. diazomethane, trimethylsilyl-diazomethane (TMSD)) | -OH, -SH<br>$-NH_2$, -NH-R<br>-COOH<br>$-CONH_2$<br>-CONH-R<br>$-COCH_2CO$-R | Wide applicability,<br>Diazomethane needs high care to dangers,<br>TMSD is safe, stable, & easy to treat |
| Esterification<br>(e.g. Fischer esterification, silylation) | Alcohols<br>Various trimethylsilyl reagents (e.g. TMSI, BSA, BSTFA, MSTFA, TMCS) | -OH, -SH<br>$-NH_2$<br>-COOH | The removal of $H_2O$<br>Wide applicability,<br>High-reactive & simple,<br>No work-up procedure |

In this study, among various derivatizations, TMS silylation was selected because of its high reactivity and simplicity that needs no work-up procedures. The utility of TMS silylation has already been proved for the target analysis by LC/MS and GC/MS [106-108]. It also has wide applicability to four functional groups such as -OH, $-NH_2$, -COOH, and $-PO_4H_2$ group [109,110]. Finally, TMS silylation was applied to suppress the peak tailing of polar lipids in SFC/MS/MS. In particular, the metal-affinity of phosphate was known as the main factor of peak tailing, and it occurs in water. However, even in SFC using not water but $CO_2$ as the mobile phase, phosphate also showed the peak tailing. In SFC, methanol was used as a modifier so that it

might cause the metal-affinity of phosphate. This can be explained by Brønsted and Lowry acid base theory. The acid-base reaction can proceed in forward direction if the acidity of acid is stronger than that of conjugate acid. Especially, the acidity of phosphate (p*K*a: 2.12, 7.21, 12.67) is stronger than that of methanol (p*K*a: 15.54) so that phosphate will be the anion in methanol. Finally, phosphate showed the metal-affinity even in SFC, and several acidic PLs having phosphate showed severe peak tailing in SFC/MS as well as LC/MS.

In Chapter 2, TMS silylation by using TMSI at 37°C for 30 min derivatized hydroxyl group and advanced the peak shape and detection sensitivity of several polar lipids. Ten polar lipids were analyzed successfully by SFC/MS/MS with TMS silylation. However, this method was insufficient to derivatize the phosphate group so that the detection sensitivity of PA was not advanced well. There was no improvement of LPE and LPG analysis by TMS silylation. Additionally, the condition of TMS silylation was not suitable for the PS analysis. These are caused by not the insufficient conditions but the limitation of TMS silylation. Finally, more effective derivatization having the simplicity and wide applicability was searched instead of advancing the conditions of TMS silylation using increased reaction time and temperature or different TMS silylation reagents.

Next, in Chapter 3, methylation that has high utility to derivatize phosphate was used for polar lipid profiling. Diazomethane is a conventional reagent for the methylation in the neutral media at room temperature [111,112]. However, this reagent is required to prepare every time to use, and it needs care due to its explosive and carcinogenic. Thus, in this study, trimethylsilyldiazomethane (TMSD) which is relatively safe, stable, and easy to treat, was used for methylation [113,114]. Especially, this method needs no catalyst and no working-up procedure so that it can escape the time-consuming and handy-error. Methylation can be applied to six PLs, six LPLs, and four SLs that have phosphate in their structure. Finally, TMSD methylation optimized at 50°C for 10 min was used successfully to improve the analysis of various polar lipids including PS and PA.

In this thesis, TMS silylation and TMSD methylation were characterized detail for polar lipid profiling. These simple and fast derivatizations were effective to advance the peak shape and detection sensitivity of polar lipids.

### 1.3.2 SFC

SFC is a chromatographic technique in which a supercritical fluid (SF) is used as the mobile phase. SF is a substance whose temperature and pressure are above the critical values (Figure 1.5), and it possesses features such as low viscosity and high diffusivity. SFC, a hybrid of GC and HPLC, incorporates many features of these two techniques [115]. SFs have densities and dissolving capacities similar to those of certain liquids, but lower viscosities and better diffusion properties [116]. SFC is applicable to thermally degradable compounds that cannot be analyzed by GC. Furthermore, the separation capacity of SFC is much higher than that of HPLC owing to the high diffusivity of the SF. According to the van Deemter curve, SFC maintains a low height equivalent to a theoretical plate (HETP) at a relatively high flow rate; therefore, the analysis time can be shortened [117]. SFC also provides a wide range of separation modes can be obtained through pressure and temperature manipulation [118]. Therefore, it exhibits suitable properties for use as the mobile phase of chromatography.

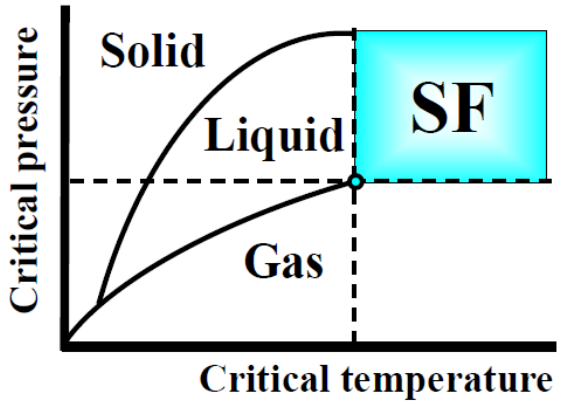


**Figure 1.5** Phase diagram of solid, liquid, gas, and supercritical fluid (SF)

Carbon dioxide ($CO_2$) is the most frequently used mobile phase in SFC because of its low critical pressure (73 atm) and low critical temperature (31.8ºC). Furthermore, $CO_2$ is chemically inert, noninflammable, relatively less toxic, and inexpensive. Supercritical carbon dioxide ($SCCO_2$) has low polarity and is hence effective for the analysis of hydrophobic compounds [119]. In addition, the polarity of the mobile phase in SFC can be changed by adding a polar organic solvent such as methanol as a modifier [120]. Therefore, $SCCO_2$ is useful for the high-throughput and simultaneous analysis of various lipids with a wide range of polarities. For example, the separation of 14 types of lipids such as PLs, glycolipids, SLs, and neutral lipids was carried out successfully by SFC [88]. In carotenoid analysis, SFC with a monolith octadecylsilylated (ODS) column allowed for the efficient separation of two carotenes and five xanthophylls [29]. Furthermore, the epoxides of carotenoids and its FA ester were separated by SFC [121]. High-throughput analysis of diverse triacylglycerols (TAGs) in a lipid mixture was obtained by SFC [122]. In addition, SFC was effective to analyze oxidized lipids such as epoxidized carotenoids [123] and oxidized PC [124]. Finally, in lipidomics, SFC is emerging as a powerful tool to separate various lipids in a mixture. In this study, SFC was used to analyze the derivatized polar lipid species.

### 1.3.3 MS/MS

For the spectrometric detection, MS/MS is useful to determine the complex species of polar lipids. MS/MS involves multiple steps of ion selection with the fragmentation of ions between the stages. Figure 1.6 shows the basic layout for a MS/MS with two mass analyzers, MS1 and MS2, separated by a collision chamber. The triple quadrupole (QqQ) MS, a common MS/MS, consists of two quadrupole mass analyzers, Q1 and Q3, separated by an ion fragmentation chamber q2. The collision of an ion with a neutral atom or molecule in q2 fragments the ion by collision-induced dissociation (CID) [89]. On QqQ MS, MRM (multiple reaction monitoring) is a non-scanning technique, in which fragmentation is the means to

increase the selectivity of analysis. MRM is used to monitor a particular product ion of a selected precursor ion. The specific pair of *m/z* values associated with the selected precursor and product ions is referred to as a "transition" and can be written as "precursor ion *m/z* > product ion *m/z*." The MRM transition has several parameters such as precursor ion *m/z*, product ion *m/z*, cone voltage (CV), and MS/MS collision energy (CE). By product ion scan, several product ions of a selected precursor ion were monitored, and one product ion was selected for MRM transition. Furthermore, the values of CV and MS/MS CE were optimized to construct the transition.

In this study, multiple transitions of MRM were used to detect various species of polar lipids. In order to fragment a lipid ion, Q1 transmits a specific precursor ion to q2. After collision, the product ions are sent to Q3 for separation based on *m/z*. The number and type of product ions will depend on the molecular structure of target compounds. One MRM transition targets one specific lipid species because a lipid molecule has particular *m/z* values of both precursor and product ions. Finally, high sensitive and high selective analysis of various polar lipids was achieved by QqQ MS.

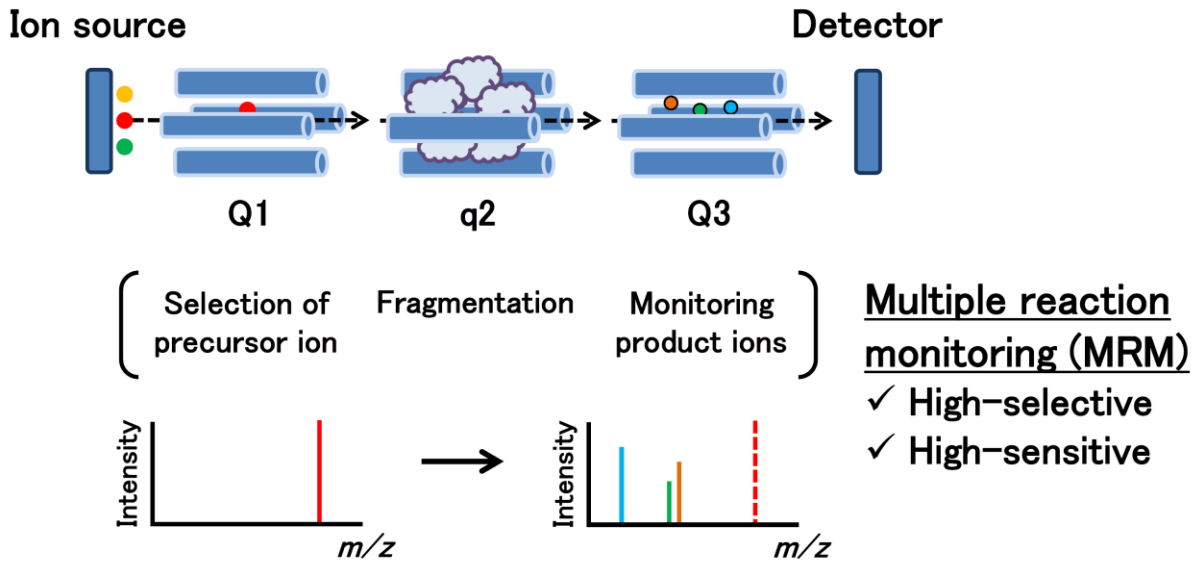


**Figure 1.6** MRM by QqQ MS

### 1.4 Objectives of this study

The overall goal of this study is to develop the high-throughput and simultaneous profiling of various polar lipids in order to obtain the detail phenotype of biological samples in lipidomics. In order to achieve the goal of this study, the strategies are set as followed.

1. Optimize the analysis conditions of SFC for the high-throughput and simultaneous analysis of various polar lipids.
2. Use silylation and methylation to improve the peak shape and detection sensitivity of several polar lipids.
3. Apply the MRM of MS/MS for the high sensitive and high selective analysis of various molecular species of polar lipids.
4. Prove the utility of developed methods by comparing the analysis data obtained from the use of both derivatization and nonderivatization.

By these strategies, an effective polar lipid profiling method was aimed to construct. Comprehensive polar lipid profiling is useful to find the alteration of each polar lipid species in biological samples and to understand the aberrant lipid metabolism in human diseases. The developed method in this study is expected to be useful for lipidomics study, especially for the study of biomarker and drug development.

This thesis is comprised of main two chapters dealing with this research subject, in which SFC/MS/MS was utilized as a main tool. In Chapter 2, a polar lipid profiling method was developed by SFC/MS/MS with TMS silylation to analyze ten polar lipids. In Chapter 3, a polar lipid profiling method based on SFC/MS/MS with TMSD methylation was developed for a high-throughput and simultaneous analysis of six PLs, six LPLs, and seven SLs.

# Chapter 2

# Development of a polar lipid profiling by SFC/MS/MS with silylation

## 2.1 Introduction

In lipidomics, an effective lipid profiling method is required to find the composition and abundance of various lipid species in biological samples, and to characterize detail the metabolic changes in human diseases [125-127]. To achieve this goal, many analytical systems for lipid profiling have developed by LC/MS [91-96] and SFC/MS [88,121-124]. However, these systems are insufficient to analyze acidic PLs such as PS and PA due to their peak tailing and low detection sensitivity. The peak tailing was caused by their metal-affinity [97,98]. Two special methods were reported to suppress the peak tailing by modifying chromatographic conditions, but several demerits limited the practical use of them [98,99]. Finally, the chemical derivatization having several merits in LC/MS [128,129] was applied to change the characteristics of analytes for obtaining the sharp peak shape. There are various derivatizations according to used reagents and targeted functional groups. PLs, LPLs, and SLs mainly have four functional groups: -OH, $-NH_2$, -COOH, and phosphate group in their structures. Several derivatizations are applicable to target these groups, and it is critical to select a suitable method for polar lipid profiling. In the selection of derivatization, the properties such as reactivity, applicability, and simplicity should be considered. The reactivity depends on the optimized conditions as well as the used reagents. On the other hand, applicability and simplicity mainly depends on the used reagents. Finally, of the applicable derivatizations to target functional groups, the priority to select a suitable method was its simplicity to escape the time-consuming and handy-error.

In this study, TMS silylation was used due to its high reactivity, simplicity, and wide applicability to four functional groups. Comparing to other methods, TMS silylation needs no work-up procedures such as additional extraction and desiccation. By silylation, the adducted TMS might block the metal-affinity to cause peak tailing and increase the hydrophobicity of compounds. Finally, the modified characteristics of silylated polar lipids are expected to be

useful to improve their peak shapes. SFC using $CO_2$ which is non-polar as mobile phase is suitable to analyze the hydrophobic compounds. MS/MS is also effective for the sensitive and selective detection of various compounds. In this chapter, an effective polar lipid profilng by SFC/MS/MS with silylation was developed to obtain sharp peak shape and high sensitivity.

## 2.2 Materials and methods

### 2.2.1 Reagents

$CO_2$ (99.9% grade; Neriki Gas, Osaka) was used as the main mobile phase. HPLC-grade methanol (Kishida Chemical, Osaka) was used as the modifier. The modifier and the make-up solvent contained 0.1% (w/w) ammonium formate (99.999%; Sigma-Aldrich, St. Louis, MO, USA). Standard samples used in this study were as follows: PG (C34:1), PA (C34:1), PI (C38:4), LPC (18:0), LPE (18:0), LPG (14:0), LPA (17:0), LPI (18:0), SM (d18:1-18:0), and So1P (d18:1) were purchased from Avanti Polar Lipids, Inc. Several TMS derivatizing reagents such as *N*-trimethylsilylimidazole (TMSI), *N, O*-bis(trimethylsilyl) acetamide (BSA), *N, O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA), *N*-methy-*N*-(trimethylsilyl) trifluoroacetamide (MSTFA), and trimethylchlorosilane (TMCS) were purchased from GL sciences, Inc.

### 2.2.2 Sample preparation

Each standard sample of polar lipids such as PG, PI, PA, LPC, LPE, LPI, LPG, LPA, SM, and So1P was prepared by dissolving in methanol. These standard solutions were stored at –30°C. Before use, each solution was diluted to the desired concentration with methanol. For the analysis of biological sample, sheep plasma was provided from Nippon Bio-Supp. Center, Tokyo, Japan. 10 μL of sheep plasma was added into 160 μL of MeOH and 0.5 μg/mL of LPC (17:0) was used as the internal standard (IS). After vortexing and incubating on ice for 10 min, the mixture was centrifuged (10000 × g, 5 min) for getting 140 μL of supernatant.

### 2.2.3 TMS silylation

Several polar lipid standards and sheep plasma were applied to TMS silylation. The extracted lipid and several polar lipid standards were desolvated by $N_2$ gas. And they were added to 60 μL of TMSI and 40 μL of pyridine and reacted at 37°C for 30 min. The silylated lipids were subjected to SFC/MS/MS.

### 2.2.4 SFC/MS/MS analysis

SFC/MS/MS analysis was performed by using Analytical SFC Method Station (Waters, Milford, MA, USA), which included a fluid delivery module, an Alias autosampler, an Analytical-2-Prep column oven, a 2998 photodiode array detector, a 3100 mass detector, an automated back pressure regulator (ABPR), and a Xevo TQ (Waters, Milford, MA, USA) mass spectrometer. The SFC/QqQ MS systems were controlled by SuperChrom automated control module software and MassLynx software, respectively. The flow rate of the mobile phase containing the modifier, back pressure, and column temperature were set to 3 mL/min, 10 MPa, and 37°C, respectively. For each run, 5 μL of the sample was injected by the full sample loop injection method. For the analysis of silylated polar lipids, the following modifier gradient conditions were employed: starting modifier, 15% (v/v); ramping to 20% (v/v) over 5 min; holding for 5 min; return to 15% (v/v) over 1 min; total run time, 11 min. On the other hand, the gradient conditions for the analysis of nonsilylated polar lipids were as follows: starting modifier, 15% (v/v); ramping to 30% (v/v) over 15 min; return to 15% (v/v) over 1 min; total run time, 16 min. QqQ MS analysis was performed in the positive ion mode of ESI, under the following conditions: capillary voltage, 3000 V; source temperature, 150°C; desolvation temperature, 350°C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 800 L/h; collision gas flow rate, 12 mL/h; MS CE, 20 V; extractor voltage, 3 V.

## 2.3 Results

### 2.3.1 Application of TMS silylation to polar lipid profiling by SFC/MS/MS

Several silylation reagents including TMSI, BSA, BSTFA, MSTFA, and TMCS were investigated to select the most effective reagent for the TMS silylation. In this test, TMSI was selected as the derivatizing reagent due to its high performance to silylate successfully ten polar lipids. The silylation facilitated the substitution of hydroxyl groups in polar lipids for trimethylsiloxy groups (Figure 2.1). Furthermore, the number of the adducted TMS group was different according to the number of the hydroxyl group in each polar lipid. The adducted TMS group numbers of each polar lipid were PG, 2; PI, 5; PA, 1; LPC, 1; LPE, 1; LPG, 3; LPA, 2; LPI, 5; SM, 1; and So1P, 2 (Figure 2.2).

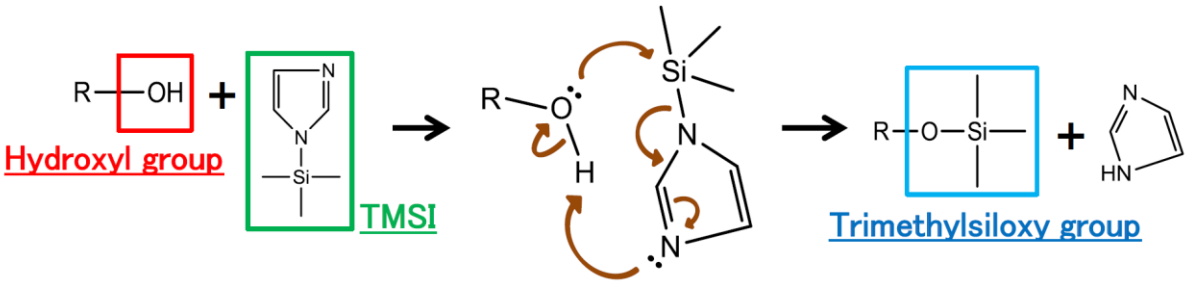


**Figure 2.1** Silylation by using TMSI

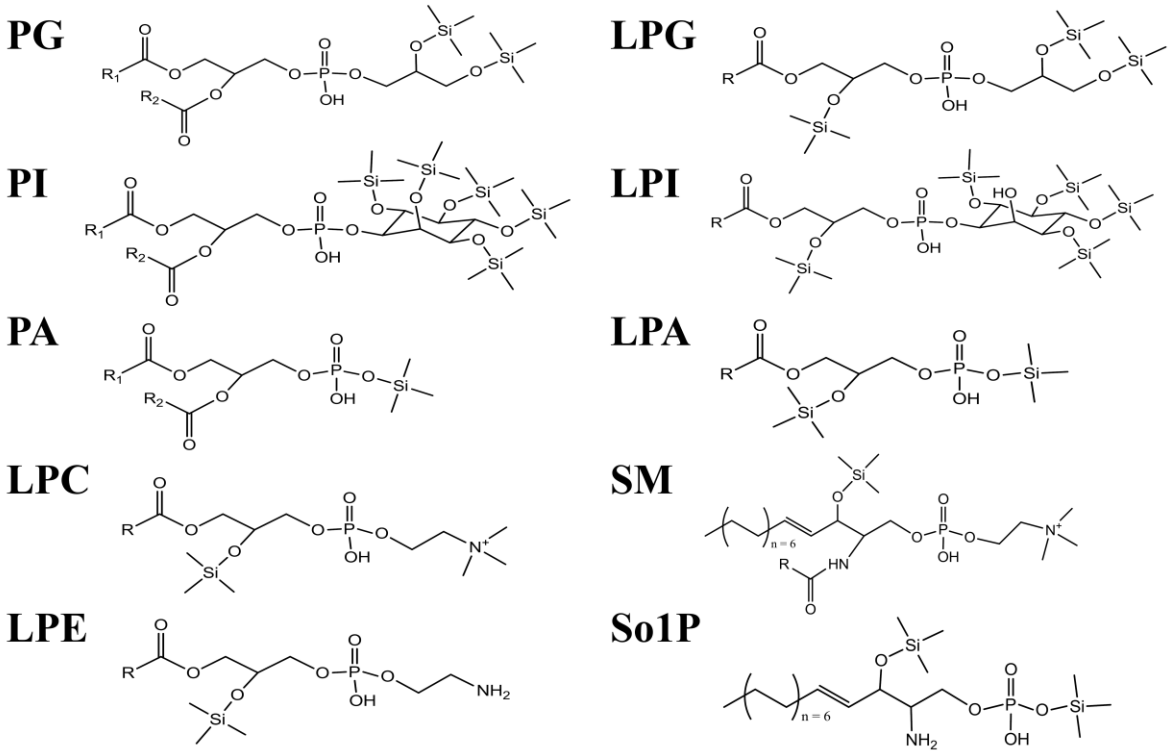


**Figure 2.2** The molecular structure of silylated polar lipids

To test the repeatability of the silylation, each polar lipid was silylated five times (n=5) respectively and analyzed by SFC/MS/MS. The relative peak area (compound/internal standard) of each compound was calculated and the variations were expressed by RSD (%). As a result, the RSD (%) of silylated polar lipids were PG, 9.6%; PI, 3.9%; PA, 6.8%; LPC, 2.1%; LPE, 7.5%; LPG, 8.8%; LPI, 9.2%; LPA, 9%; SM, 3.5%; and So1P, 2.7%. The RSDs (%) were under 10%, and it proved that the repeatability of silylation was sufficient to be used for polar lipid profiling.

In addition, the derivatization efficiency was tested to prove the utility of silylation for polar lipid profiling. The derivatization efficiency of silylated polar lipid, i.e., the percentage of silylated compounds (peak area of a silylated compound/sum of the peak areas of silylated and nonsilylated compounds × 100 (%)), was examined: PG, 99.7%; PI, 95.7%; PA, 72.1%; LPC, 99.9%; LPE, 80.5%; LPG, 99.5%; LPI, 89.4%; LPA, 99%; SM, 99.9%; and So1P, 99.5%. As a result, most polar lipids were effectively silylated (>72.1%).

In the polar lipid analysis by MS/MS, the MRM condition was optimized to analyze ten polar lipids by nonsilylation and silylation (Table 2.1). The parameters of the MRM condition consisted of MRM transitions, CV, and MS/MS CE. With nonsilylation, each polar lipid has a different *m/z* value of the precursor ion according to the kind of adducted ions, such as a hydrogen ion ($H^+$; *m/z* 1.01), an ammonium ion ($NH_4^+$; *m/z* 18.03), and a sodium ion ($Na^+$; *m/z* 22.99). Ten polar lipids were detected as $[M+H]^+$ ions in the positive ion mode by silylation. Upon the fragmentation by CID, the occurred neutral loss was different according to the number of adducted TMS groups in the polar lipids: : PG, 315 Da ($C_9H_{24}O_6PSi_2$); PI, 619 Da ($C_{21}H_{52}O_9PSi_5$); PA, 169 Da ($C_3H_{10}O_4PSi$); LPE, 140 Da ($C_2H_8NO_4P$); LPG, 315 Da ($C_9H_{24}O_6PSi_2$); LPA, 169 Da ($C_3H_{10}O_4PSi$); and LPI, 547 Da ($C_{18}H_{44}O_9PSi_4$). In the analysis of choline-containing LPC and SM, phosphoryl choline (*m/z* 184) was detected as the fragment ion. In the analysis of So1P, *m/z* 264 was detected by the loss of 269 Da ($C_6H_{20}O_5PSi_2$). We optimized the CV by comparing the peak intensities and signal-to-noise ratios (S/N) obtained from the programmed cone voltages to increments of 5 V from 15 to 45 V. Subsequently, the CE was also optimized by the same methods.

Second, several columns including Inertsil ODS-4 column (250 × 4.6 mm ID; 5 μm, GL Sciences), Berger cyano column (250 × 4.6 mm ID; 6 μm, Waters), XBridge shield RP 18 column (250 × 4.6 mm ID; 5 μm, Waters), Inertsil C8-3 column (250 × 4.6 mm ID; 5 μm, GL Sciences), Inertsil C8-4 column (250 × 4.6 mm ID; 5 μm, GL Sciences), and Inertsil ODS-EP column (250 × 4.6 mm ID; 5 μm, GL Sciences) were used to select the most effective column. Ten polar lipid standards were used to examine the candidate columns by the resolution and peak shapes. Finally, an Inertsil ODS-EP column (250 × 4.6 mm ID; 5 μm, GL Sciences) was chosen for polar lipid profiling.

**Table 2.1** Optimized MRM conditions of polar lipid profiling by nonsilylation and TMS silylation

| Polar lipids | Ion mode | MRM transitions | CV (V) | MS/MS CE (eV) |
|---|---|---|---|---|
| Nonsilylation | | | | |
| PG | Positive | $[M+NH_4] > [M+NH_4–189]$ | 25 | 20 |
| PA | Positive | $[M+NH_4] > [M+NH_4–116]$ | 30 | 25 |
| PI | Positive | [M+H] > [M+H–260] | 30 | 20 |
| LPC | Positive | [M+H] > 184 | 40 | 35 |
| LPE | Positive | [M+H] > [M+H–141] | 30 | 20 |
| LPG | Negative | [M–H] > [M–H–228] | 45 | 25 |
| LPA | Negative | [M–H] > 153 | 30 | 20 |
| LPI | Positive | [M+Na] > 283 | 45 | 30 |
| SM | Positive | [M+H] > 184 | 40 | 30 |
| So1P | Positive | [M+H] > 264 | 25 | 15 |
| TMS silylation | | | | |
| PG | Positive | [M+H] > [M+H–316] | 20 | 30 |
| PA | Positive | [M+H] > [M+H–170] | 25 | 25 |
| PI | Positive | [M+H] > [M+H–620] | 35 | 30 |
| LPC | Positive | [M+H] > 184 | 45 | 35 |
| LPE | Positive | [M+H] > [M+H–141] | 30 | 15 |
| LPG | Positive | [M+H] > [M+H–316] | 30 | 20 |
| LPA | Positive | [M+H] > [M+H–170] | 30 | 15 |
| LPI | Positive | [M+H] > [M+H–548] | 45 | 25 |
| SM | Positive | [M+H] > 184 | 45 | 30 |
| So1P | Positive | [M+H] > 264 | 25 | 20 |

Twenty MRM data of ten nonsilylated lipids and ten silylated lipids was obtained by using one optimized MRM transition to analyze one polar lipid (Figure 2.3). Nonsilylated polar lipids were analyzed with a gradient modifier from 15% to 30% (v/v) for 16 min. As a result, a peak tailing was shown in the analysis of PA, PI, LPE, LPA and LPI. Furthermore, it was difficult to find a peak of So1P due to the continuous elution detected after 7 min (Figure 2.3A). However, the peak tailings were advanced in the analysis of PA, PI, LPA, LPI, and So1P by silylation (Figure. 2.3B). The silylated polar lipids were analyzed with a gradient modifier from 15% to 20% (v/v) for 11 min.

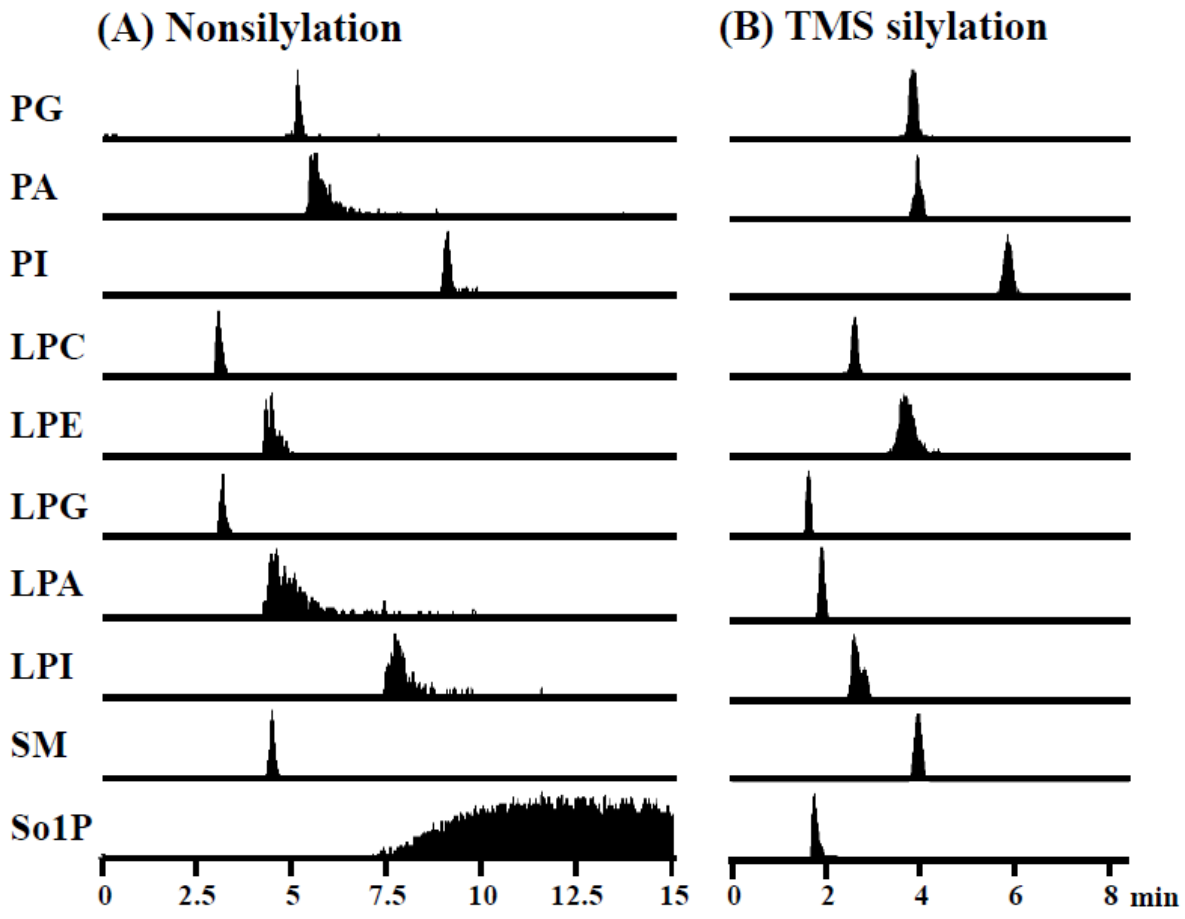


**Figure 2.3** MRM data of ten polar lipids by nonsilylation (A) and silylation (B).

### 2.3.2 Validation study

The validation study of the silylated lipid analysis was performed to estimate its analytical performance for the quantitative analysis of polar lipids (Table 2.2). The limit of detection (LOD) of the silylated lipids was also compared with the LOD of nonsilylated lipids. The standard samples of the ten polar lipids were PG (C34:1), PA (C34:1), PI (C38:4), LPC (18:0), LPE (18:0), LPG (14:0), LPA (17:0), LPI (18:0), SM (d18:1-18:0), and So1P (d18:1). In this validation study, the developed analytical method showed high reproducibility. Each polar lipid standard was analyzed six times with an IS (LPC, 17:0), and the relative standard deviation (RSD (%)) was calculated. The RSDs (%) of the relative retention times (compound/internal standard) were fewer than 2%, and the RSDs (%) of the relative peak areas (compound/internal standard) were under 10%, except in the case of PI. Furthermore, the correlation ($R^2$) of each polar lipid analysis was, at minimum, above 0.9901, and this proved the high reliability of the quantitative analysis. The LODs of the nonsilylated PG, PI, LPA, LPI, and So1P were 820, 940, 24000, 5400, and 7900 fmol, respectively. However, their LODs were decreased to 66, 23, 47, 140, and 5.3 fmol, respectively, by TMS silylation. Finally, the LODs of PG, PI, LPA, LPI, and So1P were enhanced 12-, 40-, 510-, 39-, and 1490-fold, respectively. The LODs of PA, LPC, and SM were not advanced: PA, 630 to 310; LPC, 9.4 to 7.2; SM, 25 to 9.8 fmol. In addition, the LODs of LPE and LPG increased reversely: LPE, 86 to 140; and LPG, 110 to 220 fmol. However, the increasing degree of their LODs was not high and it was shown that the application of TMS silylation is effective for polar lipid profiling. In conclusion, the developed method showed high reproducibility, high reliability, and high sensitivity.

**Table 2.2** Validation of silylated lipid analysis and the LODs comparison with nonsilylated lipid analysis

| Polar lipids[a] | RT (min) | RSD (n=6) (%)[b] RT[c] | RSD (n=6) (%)[b] Peak area[d] | Correlation ($R^2$) | Linear range (fmol) | LOD (fmol) Silylated | LOD (fmol) Nonsilylated |
|---|---|---|---|---|---|---|---|
| PG | 3.65 | 0.7 | 5.9 | 0.9999 | 66 – 33000 | 66 | 820 |
| PA | 3.75 | 0.7 | 5.5 | 0.9928 | 630 – 31000 | 310 | 630 |
| PI | 5.58 | 1.7 | 14.8 | 0.9982 | 75 – 19000 | 23 | 940 |
| LPC | 2.40 | 0.4 | 3.1 | 0.9984 | 9.4 – 47000 | 7.2 | 9.4 |
| LPE | 3.43 | 0.7 | 7.1 | 0.9988 | 170 – 8600 | 140 | 86 |
| LPG | 1.59 | 1.0 | 8.7 | 0.9993 | 220 – 11000 | 220 | 110 |
| LPA | 1.84 | 1.6 | 9.0 | 0.9912 | 95 – 24000 | 47 | 24000 |
| LPI | 2.51 | 1.1 | 5.2 | 0.9901 | 290 – 7200 | 140 | 5400 |
| SM | 3.69 | 0.8 | 6.3 | 0.9989 | 9.8 – 4900 | 9.8 | 25 |
| So1P | 1.72 | 1.7 | 8.4 | 0.9972 | 13 – 13000 | 5.3 | 7900 |

[a] Polar lipid standards used in this study were following as PG (C34:1), PA (C34:1), PI (C38:4), LPC (18:0), LPE (18:0), LPG (14:0), LPA (17:0), LPI (18:0), SM (d18:1-18:0), and So1P (d18:1).

[b] Concentration of each lipid was 5 μg/mL (PG, PA, PI, LPC), 1 μg/mL (LPE, LPA, SM), 0.5 μg/mL (LPG, LPI, So1P).

[c] Relative retention time (compound/internal standard)

[d] Relative peak area (compound/internal standard)

### 2.3.3 Analysis of a biological sample

Next, the lipids extracted from sheep plasma were analyzed to evaluate the usefulness of the developed method for simultaneous profiling of diverse polar lipids in a mixture. For the effective extraction of polar lipids from the plasma, MeOH was used as the extraction solvent [130]. In addition, LPC (17:0) was used as the IS. For the detailed analysis of each polar lipid, many MRM transitions were used to analyze various molecular species having different FA compositions. The numbers of used MRM transitions were PG, 35; PI, 44; PA, 35; LPC, 25; LPE, 19; LPG, 19; LPI, 19; LPA, 19; SM, 35; and So1P, 1 (Table 2.3). These numbers depend on the expected candidates of various polar lipids in sheep plasma. In this study, the performance of TMS silylation was compared with nonsilylation to polar lipid profiling of sheep plasma. As a result, diverse polar lipids that consist of 16 PIs, 20 LPCs, 5 LPEs, 5 LPAs, 1 LPI, 31 SMs, and So1P were successfully analyzed by the developed method applying the silylation (Table 2.4). It was also sufficient to separate each polar lipid that has specific total chain length (Cn) and total unsaturation degree (Un). The major species of PI, LPC, LPE and SM were analyzed even by nonsilylation. However, by applying the TMS silylation, twenty minor species of PI, LPC, LPE, and SM, and seven molecular species of LPA, LPI, and So1P were additionally analyzed. Finally, a more detailed qualitative analysis of major and minor polar lipids was achieved by the developed method.

In the quantitative analysis of the sheep plasma, the relative ratio of molecular species in each polar lipid was calculated. The main composition of the FAs was different, depending on the kind of polar lipid. For example, two PIs that have a fatty acids composition of C38:4 and C36:4 were mainly detected. Moreover, palmitic acid (C16:0) and stearic acid (C18:0) were the main finds in the analysis of LPC and LPE. In the case of LPA, linoleic acid (C18:2) and arachidonic acid (C20:4) were mainly detected. Nervonic acid (C24:1), palmitic acid (C16:0), and lignoceric acid (C24:0) were mainly found in the SM of the sheep plasma.

**Table 2.3** MRM transitions for polar lipid profiling of a biological sample by SFC/MS/MS with silylation

| Fatty acid composition | PG | | PI | | PA | | Fatty acid composition | SM | |
|---|---|---|---|---|---|---|---|---|---|
| Cn[a]_Un[b] | Q1[c] | Q3[d] | Q1 | Q3 | Q1 | Q3 | Cn_Un | Q1 | Q3 |
| 42:2 | | | 1307.7 | 687.4 | | | d18:1-28:0 | 943.6 | 184 |
| 42:3 | | | 1305.7 | 685.4 | | | d18:1-28:1 | 941.6 | 184 |
| 42:4 | | | 1303.7 | 683.4 | | | d18:1-28:2 | 939.6 | 184 |
| 42:5 | | | 1301.7 | 681.4 | | | d18:1-26:0 | 915.5 | 184 |
| 42:6 | | | 1299.7 | 679.4 | | | d18:1-26:1 | 913.5 | 184 |
| 42:7 | | | 1297.7 | 677.4 | | | d18:1-26:2 | 911.5 | 184 |
| 42:8 | | | 1295.7 | 675.4 | | | d18:1-26:3 | 909.5 | 184 |
| 42:9 | | | 1293.7 | 673.4 | | | d18:1-26:4 | 907.5 | 184 |
| 42:10 | | | 1291.7 | 671.4 | | | d18:1-26:5 | 905.5 | 184 |
| 40:2 | 975.5 | 659.3 | 1279.6 | 659.3 | 829.5 | 659.3 | d18:1-26:6 | 903.5 | 184 |
| 40:3 | 973.5 | 657.3 | 1277.6 | 657.3 | 827.5 | 657.3 | d18:1-24:0 | 887.5 | 184 |
| 40:4 | 971.5 | 655.3 | 1275.6 | 655.3 | 825.5 | 655.3 | d18:1-24:1 | 885.5 | 184 |
| 40:5 | 969.5 | 653.3 | 1273.6 | 653.3 | 823.5 | 653.3 | d18:1-24:2 | 883.5 | 184 |
| 40:6 | 967.5 | 651.3 | 1271.6 | 651.3 | 821.5 | 651.3 | d18:1-24:3 | 881.5 | 184 |
| 40:7 | 965.5 | 649.3 | 1269.6 | 649.3 | 819.5 | 649.3 | d18:1-24:4 | 879.5 | 184 |
| 40:8 | 963.5 | 647.3 | 1267.6 | 647.3 | 817.5 | 647.3 | d18:1-24:5 | 877.5 | 184 |
| 38:0 | 951.5 | 635.3 | 1255.6 | 635.3 | 805.5 | 635.3 | d18:1-24:6 | 875.5 | 184 |
| 38:1 | 949.5 | 633.3 | 1253.6 | 633.3 | 803.5 | 633.3 | d18:1-22:0 | 859.5 | 184 |
| 38:2 | 947.5 | 631.3 | 1251.6 | 631.3 | 801.5 | 631.3 | d18:1-22:1 | 857.5 | 184 |
| 38:3 | 945.5 | 629.3 | 1249.6 | 629.3 | 799.5 | 629.3 | d18:1-22:2 | 855.5 | 184 |
| 38:4 | 943.5 | 627.3 | 1247.6 | 627.3 | 797.5 | 627.3 | d18:1-22:3 | 853.5 | 184 |
| 38:5 | 941.5 | 625.3 | 1245.6 | 625.3 | 795.5 | 625.3 | d18:1-22:4 | 851.5 | 184 |
| 38:6 | 939.5 | 623.3 | 1243.6 | 623.3 | 793.5 | 623.3 | d18:1-22:5 | 849.5 | 184 |
| 38:7 | 937.5 | 621.3 | 1241.6 | 621.3 | 791.5 | 621.3 | d18:1-22:6 | 847.5 | 184 |
| 38:8 | 935.5 | 619.3 | 1239.6 | 619.3 | 789.5 | 619.3 | d18:1-20:0 | 831.5 | 184 |
| 36:0 | 923.5 | 607.3 | 1227.6 | 607.3 | 777.5 | 607.3 | d18:1-20:1 | 829.5 | 184 |
| 36:1 | 921.5 | 605.3 | 1225.6 | 605.3 | 775.5 | 605.3 | d18:1-20:2 | 827.5 | 184 |
| 36:2 | 919.5 | 603.3 | 1223.6 | 603.3 | 773.5 | 603.3 | d18:1-20:3 | 825.5 | 184 |
| 36:3 | 917.5 | 601.3 | 1221.6 | 601.3 | 771.5 | 601.3 | d18:1-20:4 | 823.5 | 184 |
| 36:4 | 915.5 | 599.3 | 1219.6 | 599.3 | 769.5 | 599.3 | d18:1-18:0 | 803.5 | 184 |
| 36:5 | 913.5 | 597.3 | 1217.6 | 597.3 | 767.5 | 597.3 | d18:1-18:1 | 801.5 | 184 |
| 36:6 | 911.5 | 595.3 | 1215.6 | 595.3 | 765.5 | 595.3 | d18:1-18:2 | 799.5 | 184 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 34:0 | 895.5 | 579.3 | 1199.6 | 579.3 | 749.5 | 579.3 | d18:1-16:0 | 775.5 | 184 |
| 34:1 | 893.5 | 577.3 | 1197.6 | 577.3 | 747.5 | 577.3 | d18:1-16:1 | 773.5 | 184 |
| 34:2 | 891.5 | 575.3 | 1195.6 | 575.3 | 745.5 | 575.3 | d18:1-14:0 | 747.5 | 184 |
| 34:3 | 889.5 | 573.3 | 1193.6 | 573.3 | 743.5 | 573.3 | | So1P | |
| 34:4 | 887.5 | 571.3 | 1191.6 | 571.3 | 741.5 | 571.3 | Cn_Un | Q1 | Q3 |
| 34:5 | 885.5 | 569.3 | 1189.6 | 569.3 | 739.5 | 569.3 | d18:1 | 524.1 | 264 |
| 34:6 | 883.5 | 567.3 | 1187.6 | 567.3 | 737.5 | 567.3 | | | |
| 32:0 | 867.5 | 551.3 | 1171.6 | 551.3 | 721.5 | 551.3 | | | |
| 32:1 | 865.5 | 549.3 | 1169.6 | 549.3 | 719.5 | 549.3 | | | |
| 32:2 | 863.5 | 547.3 | 1167.6 | 547.3 | 717.5 | 547.3 | | | |
| 32:3 | 861.5 | 545.3 | 1165.6 | 545.3 | 715.5 | 545.3 | | | |
| 32:4 | 859.5 | 543.3 | 1163.6 | 543.3 | 713.5 | 543.3 | | | |

[a] Cn; total chain length

[b] Un; total degree of unsaturation

[c] Q1; precursor *m/z*

[d] Q3; fragment *m/z*

**Table 2.3** (Continued)

| Fatty acid composition | LPC | | LPE | | LPG | | LPI | | LPA | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cn_Un | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 |
| 24:2 | 676.3 | 184 | | | | | | | | |
| 24:3 | 674.3 | 184 | | | | | | | | |
| 24:4 | 672.3 | 184 | | | | | | | | |
| 24:5 | 670.3 | 184 | | | | | | | | |
| 24:6 | 668.3 | 184 | | | | | | | | |
| 24:7 | 666.3 | 184 | | | | | | | | |
| 22:1 | 650.2 | 184 | 608.1 | 467.1 | 783.1 | 467.1 | 1015.3 | 467.1 | 637.3 | 467.1 |
| 22:2 | 648.2 | 184 | 606.1 | 465.1 | 781.1 | 465.1 | 1013.3 | 465.1 | 635.3 | 465.1 |
| 22:3 | 646.2 | 184 | 604.1 | 463.1 | 779.1 | 463.1 | 1011.3 | 463.1 | 633.3 | 463.1 |
| 22:4 | 644.2 | 184 | 602.1 | 461.1 | 777.1 | 461.1 | 1009.3 | 461.1 | 631.3 | 461.1 |
| 22:5 | 642.2 | 184 | 600.1 | 459.1 | 775.1 | 459.1 | 1007.3 | 459.1 | 629.3 | 459.1 |
| 22:6 | 640.2 | 184 | 598.1 | 457.1 | 773.1 | 457.1 | 1005.3 | 457.1 | 627.3 | 457.1 |
| 20:0 | 624.2 | 184 | 582.1 | 441.1 | 757.1 | 441.1 | 989.3 | 441.1 | 611.3 | 441.1 |
| 20:1 | 622.2 | 184 | 580.1 | 439.1 | 755.1 | 439.1 | 987.3 | 439.1 | 609.3 | 439.1 |
| 20:2 | 620.2 | 184 | 578.1 | 437.1 | 753.1 | 437.1 | 985.3 | 437.1 | 607.3 | 437.1 |
| 20:3 | 618.2 | 184 | 576.1 | 435.1 | 751.1 | 435.1 | 983.3 | 435.1 | 605.3 | 435.1 |
| 20:4 | 616.2 | 184 | 574.1 | 433.1 | 749.1 | 433.1 | 981.3 | 433.1 | 603.3 | 433.1 |
| 18:0 | 596.2 | 184 | 554.1 | 413.1 | 729.1 | 413.1 | 961.3 | 413.1 | 583.3 | 413.1 |
| 18:1 | 594.2 | 184 | 552.1 | 411.1 | 727.1 | 411.1 | 959.3 | 411.1 | 581.3 | 411.1 |
| 18:2 | 592.2 | 184 | 550.1 | 409.1 | 725.1 | 409.1 | 957.3 | 409.1 | 579.3 | 409.1 |
| 18:3 | 590.2 | 184 | 548.1 | 407.1 | 723.1 | 407.1 | 955.3 | 407.1 | 577.3 | 407.1 |
| 16:0 | 568.2 | 184 | 526.1 | 385.1 | 701.1 | 385.1 | 933.3 | 385.1 | 555.3 | 385.1 |
| 16:1 | 566.2 | 184 | 524.1 | 383.1 | 699.1 | 383.1 | 931.3 | 383.1 | 553.3 | 383.1 |
| 16:2 | 564.2 | 184 | 522.1 | 381.1 | 697.1 | 381.1 | 929.3 | 381.1 | 551.3 | 381.1 |
| 14:0 | 540.2 | 184 | 498.1 | 357.1 | 673.1 | 357.1 | 905.3 | 357.1 | 527.3 | 357.1 |

**Table 2.4** Polar lipid profiling of sheep plasma by SFC/MS/MS with TMS silylation and the comparison with nonsilylation

| Polar lipids | Cn_Un | TMS silylation | | Nonsilylation | |
|---|---|---|---|---|---|
| | | RT[a] (min) | Relative area[b] (%) | RT[a] (min) | Detection |
| PI | 40:4 | 6.43 | (0.06 ± 0.11)[c] | | N.D.[d] |
| | 40:5 | 6.23 | 1.2 ± 0.6 | | N.D. |
| | 40:6 | 6.07 | 1.7 ± 0.3 | 8.82 | |
| | 38:2 | 6.21 | (0.2 ± 0.2) | | N.D. |
| | 38:3 | 5.92 | 5 ± 2. | 8.97 | |
| | 38:4 | 5.71 | 64 ± 2. | 8.71 | |
| | 38:5 | 5.47 | 2.7 ± 0.5 | 8.56 | |
| | 38:6 | 5.24 | 0.7 ± 0.4 | | N.D. |
| | 36:1 | 5.82 | (0.1 ± 0.1) | | N.D. |
| | 36:2 | 5.53 | 4.3 ± 0.5 | | N.D. |
| | 36:3 | 5.16 | 1.9 ± 0.5 | 8.16 | |
| | 36:4 | 4.91 | 11 ± 1. | 8.13 | |
| | 36:5 | 4.87 | (0.02 ± 0.04) | | N.D. |
| | 34:1 | 4.97 | 0.7 ± 0.4 | | N.D. |
| | 34:2 | 4.76 | 6 ± 2. | | N.D. |
| | 32:1 | 4.41 | (0.02 ± 0.03) | | N.D. |
| LPC | 24:4 | 2.86 | (0.0008 ± 0.0013) | | N.D. |
| | 24:5 | 2.77 | (0.002 ± 0.004) | 3.33 | |
| | 24:6 | 2.61 | (0.002 ± 0.003) | 3.19 | |
| | 22:1 | 3.01 | 0.03 ± 0.01 | 3.62 | |
| | 22:2 | 2.83 | (0.001 ± 0.001) | 3.41 | |
| | 22:5 | 2.34 | 0.62 ± 0.05 | 2.99 | |
| | 22:6 | 2.28 | 1.8 ± 0.2 | 2.93 | |
| | 20:0 | 2.71 | 0.22 ± 0.07 | 3.38 | |
| | 20:1 | 2.52 | 0.8 ± 0.1 | 3.12 | |
| | 20:2 | 2.39 | 0.7 ± 0.1 | 2.99 | |
| | 20:3 | 2.21 | 1.3 ± 0.1 | 2.81 | |
| | 20:4 | 2.10 | 4.2 ± 0.2 | 2.72 | |
| | 18:0 | 2.31 | 38.8 ± 0.4 | 2.95 | |
| | 18:1 | 2.14 | 10.13 ± 0.04 | 2.72 | |
| | 18:2 | 2.01 | 11.4 ± 0.2 | 2.59 | |
| | 18:3 | 1.97 | 0.05 ± 0.02 | 2.51 | |
| | 16:0 | 1.94 | 29.4 ± 0.1 | 2.54 | |

| | | | | | |
|---|---|---|---|---|---|
| | 16:1 | 1.83 | 0.52 ± 0.04 | 2.40 | |
| | 16:2 | 1.75 | 0.002 ± 0.002 | | N.D. |
| | 14:0 | 1.72 | 0.11 ± 0.03 | 2.18 | |
| LPE | 20:4 | 3.05 | (4 ± 3) | | N.D. |
| | 18:0 | 3.72 | 54 ± 3. | 4.62 | |
| | 18:1 | 3.57 | (5 ± 2) | | N.D. |
| | 18:2 | 3.42 | (2 ± 2) | 4.21 | |
| | 16:0 | 3.12 | 35 ± 2. | 3.86 | |
| LPA | 20:4 | 1.74 | 15.1 ± 0.3 | | N.D. |
| | 18:0 | 1.89 | 4 ± 7. | | N.D. |
| | 18:1 | 1.71 | 6 ± 5. | | N.D. |
| | 18:2 | 1.69 | 70 ± 3. | | N.D. |
| | 16:0 | 1.62 | 5 ± 4. | | N.D. |
| LPI | 18:0 | 2.61 | 100. | | N.D. |
| SM | 26:0 | 6.70 | 0.11 ± 0.05 | 7.12 | |
| | 26:1 | 6.26 | 0.38 ± 0.07 | 6.83 | |
| | 26:2 | 5.96 | 0.06 ± 0.04 | 6.59 | |
| | 26:3 | 5.63 | (0.001 ± 0.002) | | N.D. |
| | 26:4 | 5.32 | (0.003 ± 0.003) | | N.D. |
| | 26:5 | 5.02 | (0.004 ± 0.006) | 6.21 | |
| | 24:0 | 5.88 | 18.1 ± 0.4 | 6.49 | |
| | 24:1 | 5.48 | 29.3 ± 0.3 | 5.99 | |
| | 24:2 | 5.24 | 7.2 ± 0.4 | 5.75 | |
| | 24:3 | 5.02 | 0.53 ± 0.02 | 5.55 | |
| | 24:4 | 4.57 | 0.06 ± 0.02 | 5.27 | |
| | 24:5 | 4.36 | 0.13 ± 0.08 | 5.15 | |
| | 24:6 | 4.19 | 0.08 ± 0.03 | 4.96 | |
| | 22:0 | 5.13 | 7.1 ± 0.4 | 5.71 | |
| | 22:1 | 4.85 | 2.7 ± 0.2 | 5.40 | |
| | 22:2 | 4.50 | 0.40 ± 0.08 | 5.16 | |
| | 22:3 | 4.31 | (0.01 ± 0.01) | 4.86 | |
| | 22:4 | 4.04 | (0.003 ± 0.004) | | N.D. |
| | 22:5 | 3.90 | 0.05 ± 0.03 | | N.D. |
| | 22:6 | 3.74 | 0.06 ± 0.03 | | N.D. |
| | 20:0 | 4.68 | 2.3 ± 0.1 | 5.07 | |
| | 20:1 | 4.21 | (0.33 ± 0.03) | 4.88 | |
| | 20:2 | 3.85 | (0.009 ± 0.004) | 4.62 | |
| | 20:3 | 3.70 | (0.003 ± 0.001) | | N.D. |

| | | | | | |
|---|---|---|---|---|---|
| | 20:4 | 3.61 | 0.004 ± 0.006 | 4.32 | |
| | 18:0 | 3.80 | 2.4 ± 0.1 | 4.50 | |
| | 18:1 | 3.64 | 0.63 ± 0.07 | 4.33 | |
| | 18:2 | 3.32 | (0.011 ± 0.008) | 4.07 | |
| | 16:0 | 3.26 | 25.8 ± 0.5 | 3.98 | |
| | 16:1 | 3.12 | 2.1 ± 0.1 | 3.81 | |
| | 14:0 | 2.79 | 0.20 ± 0.02 | 3.52 | |
| So1P | 18:1 | 1.79 | 100. | | N.D. |

[a] Average retention time (n=3)

[b] Percentage of each molecular species to each polar lipid (peak area/total peak area), the values of percentages are means±SD, (n=3)

[c] Marked by a round bracket, below the limit of quantification (LOQ)

[d] N.D.; no detection

## 2.4 Discussion

This study was the first attempt to develop a polar lipid profiling method based on SFC/MS/MS with silylation. Above all, it was the most important to select a suitable reagent for effective TMS silylation. There are various reagents for TMS silylation, and each reagent has different property and performance. In this study, several reagents including TMSI, BSA, BSTFA, MSTFA, and TMCS were investigated, and TMSI was selected because of its high reactivity. The silylation conditions by TMSI are optimized as reacting at 37°C for 30 min. Other reagents need higher temperature or longer time for silylation. According to the target compounds, not only a single reagent but also mixed two or three reagents has been widely used for silylation in the previous studies. However, a single TMSI was enough to silylate the hydroxyl groups in polar lipids, and as a result, the hydrophobicity of silylated polar lipids was increased. This changed the characters of silylated compounds on the stationary phase of the ODS column, and the retention time (RT) of polar lipids was increased. Additionally, the peak shape of several polar lipids was advanced due to the constant interaction and separation of polar lipids with the stationary phase.

To analyze the silylated polar lipids, it was necessary to optimize the MS/MS method and to find the best conditions such as column for the separation by SFC. MRM is a highly selective nonscanning technique to monitor a particular product ion of a selected precursor ion [131]. Since a lipid mixture includes several compounds having the same *m/z* values but different molecular structures, it is difficult to identify complex lipids by a single MS cycle. In MS/MS, each lipid species affords a specific product ion by CID; therefore, MRM is an effective method by which various lipids can be analyzed by selecting specific *m/z* values of the precursor and product ions. Especially, several parts such as head group, the neutral loss of head group, and the sphingoid base of SL were detected as the product ions. One polar lipid species having specific *m/z* values of the precursor and product ions was detected by one MRM transition. Therefore, by MRM analysis, it was possible to identify various polar lipids

having different head groups, FA compositions, and the sphingoid base in the case of SL. FA composition was characterized by both Cn and Un that also indicate the number of carbons and double bonds in FAs respectively. Finally, MRM provided the high selective and sensitive detection of each polar lipid species.

For the effective separation of various polar lipid species, SFC conditions such as the used column and modifier ratio were optimized. In the analysis of silylated polar lipids, not only SFC but also LC can be used. Especially, SFC has been proved as being effective for the analysis of hydrophobic compounds. SFC also provides the high-throughput and simultaneous analysis of diverse lipids [88]. Thus, SFC was used to analyze the silylated polar lipids. In the column test, Inertsil ODS-EP column (250 × 4.6 mm ID; 5 μm, GL Sciences) were selected for the polar lipid profiling. This column has a polar functional group embedded between the silica surface and the ODS group, and it is suitable for the separation of polar and nonpolar compounds. This column showed the effective separation of each polar lipid class comparing to other columns. This column separated various polar lipid species having different Cn and Un, and the lipid with a higher Cn and a lower Un represented the longer RT. The optimized modifier ratio (15-20%, 15 min) also provided the high-throughput, high-resolution analysis.

In the validation study, the repeatability of silylated polar lipids was described in terms of RSD (%). As a result, the developed method showed high reproducibility in both TMS silylation and analysis system that provides RT and peak area of same standard sample. This method also represented high reliability and sensitivity. In the examination of LOD, the detection sensitivity of PG, PI, LPI, LPA, and So1P improved upon silylation, but those of other lipids changed slightly. There was no improvement in the analysis of PA, LPC, LPE, LPG, and SM. In particular, the LODs of LPE and LPG were increased reversely by TMS silylation. This result might be caused by not the used conditions of TMS silylation but the lowered ionization efficiency. In the ESI, the hydroxyl group of compounds is easily ionized. Upon TMS silylation, the hydroxyl group of polar lipids was blocked so that the ionization

efficiency of not only LPE and LPG but also other lipids might be lowered. The peak shape of other polar lipids was advanced by TMS silylation so that their detection sensitivity improved despite the lowered ionization efficiency. However, LPE and LPG normally have the sharp peak shape by nonsilylation so that the LOD of LPE and LPG was increased by silylation. The degree of increased LODs was not high and their peak shape and intensity were also good. Finally, TMS silylation was applied to analyze simultaneously ten polar lipids: PG, PI, PA, LPC, LPE, LPG, LPI, LPA, SM, and So1P with sharp peak shape and high sensitivity. TMS silylation efficiency of ten polar lipids was also examined to confirm whether the silylation is applicable for polar lipid profiling. According to the molecular structure, the reaction to silylation by TMSI is different, and it is related to the silylation efficiency. As a result, ten polar lipids were silylated with relatively high efficiency (>72.1%).

In order to prove the utility of this method, the analysis of a biological sample (sheep plasma) was performed. The MeOH extraction was simple and easy to extract the polar lipids from the sheep plasma. A number of MRM transitions were used to detect various polar lipid species according to the head group and FA composition. High-throughput analysis of 79 polar lipid species was achieved by SFC in 7 min. By this method, not only high-abundance but also low-abundance lipid species were quantified. As a result, more 27 polar lipid species were analyzed additionally than using nonsilylation. Especially, in the PI analysis, more 10 species were analyzed upon silylation. Finally, the utility of the developed method based on SFC/MS/MS with silylation was proved to analyze ten polar lipids, especially for PI, in a biological sample.

However, TMS silylation was insufficient to improve the analysis of PA and PS. The phosphate of PA has two free hydroxyl groups. By TMSI, one of two hydroxyl groups was silylated well. However, another hydroxyl group was not silylated due to the steric hindrance. TMS is too big to be adducted to the second hydroxyl group. As a result, the peak tailing of PA was suppressed by TMS silylation, but the detection sensitivity was not advanced much.

Furthermore, PS was not silylated by TMSI at 37°C for 30 min. To silylate PS, it is required to advance the silylation conditions using the different reagent or the higher temperature and longer time. However, it was already found that TMS silylation is insufficient for PA analysis due to its steric hindrance. TMS silylation also decreased the detection sensitivity of LPE and LPG due to the lowered ionization efficiency. Therefore, for the effective polar lipid profiling, it is better to search more suitable derivatization than to optimize newly the TMS silylation.

## 2.5 Conclusion

In this study, a simultaneous profiling method for ten polar lipids by SFC/MS/MS with silylation was successfully established. Upon TMS silylation, the peak shape and detection sensitivity of several polar lipids improved. In a mixture, ten polar lipids were separated with sufficient resolution by the selected ODS column with a polar functional group. The MRM condition was also optimized to detect the silylated polar lipids. In the validation study, the developed method showed high reproducibility, high reliability, and high sensitivity. Next, in the analysis of a biological sample (sheep plasma), high-abundance and low-abundance lipid species were quantified. Various polar lipid species (16 PIs, 20 LPCs, 5 LPEs, 5 LPAs, 1 LPI, 31 SMs, and So1P) were analyzed in 7 min without purification. Comparing to nonsilylation, 27 polar lipid species, especially for 10 PI species, were analyzed additionally by silylation. Finally, by this method, the detailed characterization of polar lipids in a biological sample was achieved by the qualitative and quantitative analysis. Thus, in lipidomics, it will be expected to contribute to the effective profiling of polar lipids, especially for PI.

# Chapter 3

# Development of a polar lipid profiling by SFC/MS/MS with methylation

## 3.1 Introduction

Lipidomics has relied on the performance of lipid profiling methods so that it is critical to develop an effective analytical method. In particular, the techniques of chromatographic separation and spectrometric detection were effective for lipid profiling [132,133]. In particular, simultaneous analysis of polar lipids with various structures and polarities is very difficult. The accurate analysis of low-abundance acidic PLs such as PS and PA is hindered by severe peak tailing and low detection sensitivity. In the previous reports, adjusted mobile phase using water and phosphoric acid [98] or EDTA-prewashed column [99] were used to suppress the peak tailing of PS and PA. However, these methods showed several demerits, and the derivatization was used to develop a more effective method to suppress the peak tailing.

In our previous study, TMS silylation was used to improve the peak shape and detection sensitivity of several polar lipids [134]. It was useful to derivatize hydroxyl groups so that the hydrophobicity of polar lipids increased and SFC was effective to analyze them. However, TMS silylation was insufficient to derivatize the phosphate, and PA analysis was not advanced well. Therefore, more effective derivatization is required for polar lipid profiling. Of various derivatization methods, methylation is an effective method to derivatize the phosphate group. Diazomethane is a conventional reagent for methylation, but it should be prepared every time and needs care due to its dangerous. In the prior report, Clark *et al.* [135] used TMSD to quantify phosphatidylinositol(3,4,5)-trisphosphate (PtdIns(3,4,5)$P_3$) in cells and tissues. Using TMSD allowed for rapid and complete methylation of the phosphate groups and thus enhanced the efficiency of PtdIns(3,4,5)$P_3$ analysis. Methylation by TMSD is also applicable to analyze the compounds having phosphate groups. Various polar lipids such as six PLs, six LPLs, and four SLs contain the phosphate. Finally, in this chapter, SFC/MS/MS with methylation was used to develop the comprehensive polar lipid profiling with sharp peak shape and high sensitivity.

### 3.2 Materials and methods

#### 3.2.1 Reagents

Carbon dioxide (99.9% grade; Neriki Gas, Osaka, Japan) was used as the mobile phase. HPLC-grade methanol (Kishida Chemical, Osaka, Japan) containing 0.1% (w/w) ammonium formate (99.99%; Sigma-Aldrich, Milwaukee, WI, USA) was used as the modifier. Details of the polar lipid standards (Avanti Polar Lipids, Inc.) are as follows: PC (12:0-12:0), PC (12:0-13:0), PE (12:0-13:0), PS (12:0-13:0), PG (12:0-13:0), PI (12:0-13:0), PA (12:0-13:0), LPC (17:0), LPE (14:0), LPS (16:0), LPG (14:0), LPI (18:1), LPA (14:0), SM (d18:1-17:0), Cer (d18:1-17:0), Cer1P (d18:1-12:0), Cer1P (d18:1-16:0), So (d17:1), So1P (d17:1), Sa (d17:0), and Sa1P (d17:0). TMSD was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan).

#### 3.2.2 Sample preparation

Methanol solutions of the polar lipid standards (1 μmol/L) were prepared, stored at –30°C, and then diluted to the desired concentration. Male C57BL/6J mice were sacrificed under anesthesia, and then the livers were obtained quickly. The perfused livers were prepared with normal saline, and were kept at –80°C before use. 10 mg of mouse liver immersed in liquid $N_2$ was homogenized in a ball mill mixer MM301 (Retsch, Haan, Germany) for 1 min at 20 Hz. The homogenate was spiked with 1000 μL of IS solution (0.1 μmol/L of PC (12:0-13:0), PE (12:0-13:0), PS (12:0-13:0), PG (12:0-13:0), PI (12:0-13:0), PA (12:0-13:0), LPC (17:0), SM (d18:0-17:0), Cer (d18:1-17:0), Cer1P (d18:1-12:0), So (d17:1), So1P (d17:1), Sa (d17:0), and Sa1P (d17:0) in methanol). After vortexing and incubating on ice for 10 min, the mixture was centrifuged (10000 × g, 5 min). Finally, 900 μL of the supernatant was used for polar lipid profiling.

### 3.2.3 TMSD methylation

TMSD shows acute toxicity for inhalation, causing central nervous system depression, drowsiness, dizziness, and lung damage. Although this reagent is not explosive like diazomethane, extreme care should be taken when handing it. For this reason, methylation by TMSD has been carried out in a fume hood, with the use of adequate personal safety equipment. A solution of TMSD (2 mol/L) in hexane (50 μL) was added to the lipid extracts (100 μL) from the mouse liver and the lipid standard samples (300 μL) dissolved in methanol to obtain yellow-colored solutions. After vortexing for 30 s, methylation was performed at 50°C for 10 min (optimized conditions). Addition of glacial acetic acid (6 μL) quenched the methylation and afforded colorless samples, which were then subjected to SFC/MS/MS.

### 3.2.4 SFC/MS/MS analysis

SFC/MS/MS analysis was performed by using Analytical SFC Method Station (Waters, Milford, MA, USA), which included a fluid delivery module, an Alias autosampler, an Analytical-2-Prep column oven, a 2998 photodiode array detector, a 3100 mass detector, an ABPR, and a Xevo TQ (Waters, Milford, MA, USA) mass spectrometer. The SFC/QqQ MS systems were controlled by SuperChrom automated control module software and MassLynx software, respectively. The flow rate of the mobile phase containing the modifier, back pressure, and column temperature were set to 3 mL/min, 10 MPa, and 37°C, respectively. For each run, 5 μL of the sample was injected by the full sample loop injection method. For the analysis of methylated polar lipids, the following modifier gradient conditions were employed: starting modifier, 20% (v/v); ramping to 25% (v/v) over 5 min; return to 20% (v/v) over 1 min; total run time, 6 min. On the other hand, the gradient conditions for the analysis of nonmethylated polar lipids were as follows: starting modifier, 20% (v/v); ramping to 25% (v/v) over 5 min; holding for 5 min; return to 20% (v/v) over 1 min; total run time, 11 min. QqQ MS analysis was performed in the positive ion mode of ESI, under the following

conditions: capillary voltage, 3000 V; source temperature, 150°C; desolvation temperature, 350°C; cone gas flow rate, 50 L/h; desolvation gas flow rate, 800 L/h; collision gas flow rate, 12 mL/h; MS CE, 20 V; extractor voltage, 3 V.

### 3.3 Results

#### 3.3.1 Application of TMSD methylation to polar lipid profiling by SFC/MS/MS

TMSD was used for methylation of the phosphate groups in polar lipids (Figure 3.1). The number of adducted methoxy groups differed with the type of lipid: PC, 1; PE, 1; PS, 2; PG, 1; PI, 1; PA, 2; LPC, 1; LPE, 1; LPS, 2; LPG, 1; LPI, 1; LPA, 2; SM, 1; Cer1P, 2; So1P, 2; Sa1P, 2 (Figure 3.2). Cer, So, and Sa, which have no phosphate group, were not methylated. Lipid standards were used for each of the lipids to decide the MS/MS conditions. By ESI, sixteen methylated and three nonmethylated polar lipids were detected as $[M+H]^+$ ions in the positive ion mode.

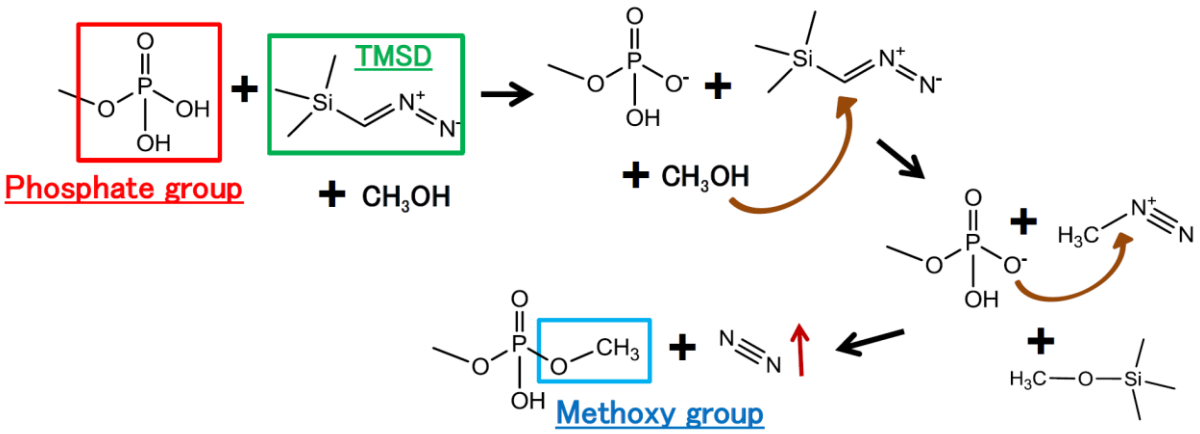


**Figure 3.1** Methylation by using TMSD

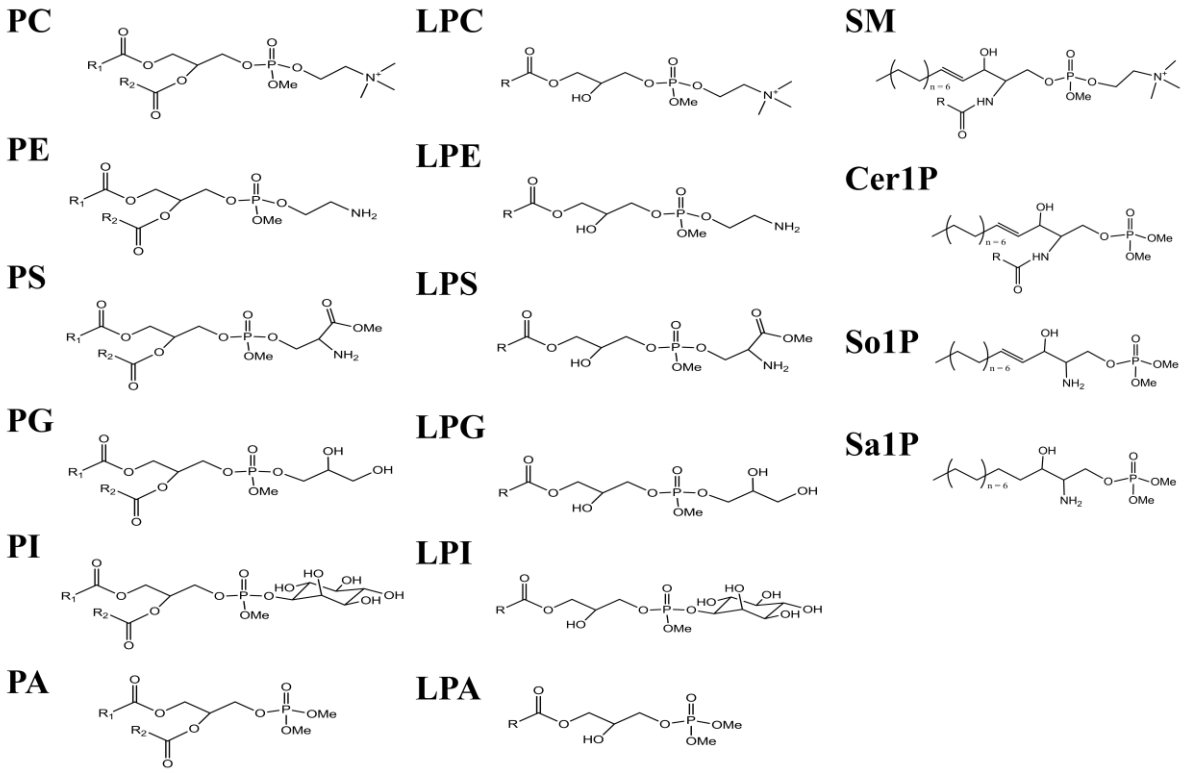


**Figure 3.2** The molecular structure of methylated polar lipids

By product ion scan, a precursor ion and fragment ions were detected in the MS/MS spectrum (Figure 3.3), and the product ion with the highest intensity was selected for the MRM transition. The neutral loss during CID differed for different polar lipids: PE and LPE, 155 Da ($C_3H_{11}NO_4P$); PS and LPS, 213 Da ($C_5H_{12}NO_6P$); PG and LPG, 186 Da ($C_4H_{11}O_6P$); PA and LPA, 126 Da ($C_2H_7O_4P$); and PI, 274 Da ($C_7H_{15}O_9P$). Methylated phosphorylcholine (*m/z* 198) was detected as the product ion in the analysis of PC, LPC, and SM, while methylated inositol monophosphate adducted by $Na^+$ (*m/z* 297) was the fragment ion in LPI analysis. The fragment ion in the analysis of Cer, Cer1P, and So1P was the sphingoid base. For example, in the case of d17:1 and d18:1, the fragment ions were *m/z* 250 and *m/z* 264, respectively. The fragment ion of Sa1P, too, was the sphingoid base: d17:0, *m/z* 252 and d18:0, *m/z* 266. The neutral loss of So and Sa was 18 Da ($H_2O$). Finally, each MRM transition for a polar lipid was optimized. Subsequently, the CV and MS/MS CE values were optimized by

comparing the peak intensities and S/Ns obtained from the programmed CVs, which ranged from 15 to 45 V (Table 3.1).

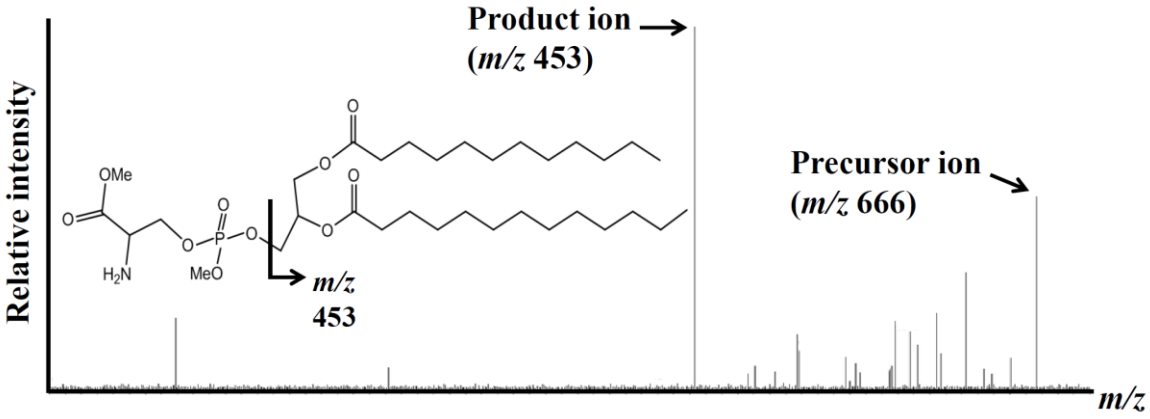


**Figure 3.3** MS/MS spectrum data of methylated PS (12:0-13:0, *m/z* 666) by product ion scan (MS/MS CE, 15eV)

**Table 3.1** Optimized MRM conditions of polar lipid profiling by methylation

| Polar lipids | Ion mode | MRM transitions | CV (V) | MS/MS CE (eV) |
|---|---|---|---|---|
| PC | Positive | [M+H] > 198 | 40 | 26 |
| PE | Positive | [M+H] > [M+H–155] | 27 | 20 |
| PS | Positive | [M+H] > [M+H–213] | 26 | 22 |
| PG | Positive | [M+H] > [M+H–186] | 24 | 18 |
| PI | Positive | [M+H] > [M+H–274] | 27 | 26 |
| PA | Positive | [M+H] > [M+H–126] | 22 | 18 |
| LPC | Positive | [M+H] > 198 | 38 | 27 |
| LPE | Positive | [M+H] > [M+H–155] | 23 | 18 |
| LPS | Positive | [M+H] > [M+H–213] | 24 | 21 |
| LPG | Positive | [M+H] > [M+H–186] | 19 | 16 |
| LPI | Positive | [M+Na] > 297 | 40 | 28 |
| LPA | Positive | [M+H] > [M+H–126] | 17 | 16 |
| SM | Positive | [M+H] > 198 | 36 | 29 |
| Cer1P | Positive | [M+H] > 264 | 22 | 33 |
| So1P | Positive | [M+H] > 264 | 22 | 15 |
| Sa1P | Positive | [M+H] > 266 | 22 | 20 |

Next, the reaction temperature (tested: 10, 20, 30, 40, 50, and 60°C) and time (tested: 5, 10, 15, and 20 min) for TMSD methylation were optimized, and each polar lipid standard was methylated three times (n=3) under the tested conditions. PC (12:0-12:0) was used as the IS, and the relative peak area (compound/internal standard) was examined, and the methylation was optimized at 50°C for 10 min. The methylation efficiency of each polar lipid, i.e., the percentage of methylated compounds (peak area of a methylated compound/sum of the peak areas of methylated and nonmethylated compounds × 100 (%)), was also examined (Table 3.2). Most polar lipids were effectively methylated (>74.9%). However, choline-containing polar lipids were methylated with low efficiency: PC, 32.3%; LPC, 37.9%; and SM, 32.9%. In order to test the repeatability of methylation, each polar lipid was methylated six times (n=6) under the optimized conditions. The variations in the relative peak areas were expressed in terms of the RSD (%): PC, 7.5%; PE, 3.1%; PS, 3.3%; PG, 7.9%; PI, 6.4%; PA, 5.7%; LPC, 7.7%; LPE, 5.1%; LPS, 4.0%; LPG, 6.2%; LPI, 7.4%; LPA, 6.9%; SM, 6.4%; Cer1P, 7.6%; So1P, 6.7%; and Sa1P, 6.6%.

**Table 3.2** Methylation efficiency of sixteen polar lipids in a mixture

| Polar lipids | The percentage of methylated compounds (%)[a] | | |
|---|---|---|---|
| | 0 Me[b] | 1 Me | 2 Me |
| PC | 67.7 ± 4.4[c] | 32.3 ± 4.4 | - |
| PE | 0.5 ± 0.4 | 99.5 ± 0.4 | - |
| PS | 0.9 ± 0.1 | 0.7 ± 0.1 | 98.4 ± 0.2 |
| PG | 6.8 ± 0.1 | 93.2 ± 0.1 | - |
| PI | 7 ± 0.5 | 93 ± 0.5 | - |
| PA | 0.2 ± 0.2 | 3 ± 0.6 | 96.8 ± 0.8 |
| LPC | 62.1 ± 7.7 | 37.9 ± 7.7 | - |
| LPE | 0.9 ± 0.1 | 99.1 ± 0.1 | - |
| LPS | 2.9 ± 0.5 | 1.4 ± 0.1 | 95.7 ± 0.6 |
| LPG | 7 ± 0.2 | 93 ± 0.2 | - |
| LPI | 1.1 ± 0.3 | 98.9 ± 0.3 | - |
| LPA | 0.2 ± 0.1 | 24.9 ± 5.8 | 74.9 ± 5.9 |
| SM | 67.1 ± 5.8 | 32.9 ± 5.8 | - |
| Cer1P | 1.1 ± 0.1 | 5.1 ± 0.1 | 93.8 ± 0.2 |
| So1P | 19 ± 0.5 | 2.3 ± 0.5 | 78.7 ± 1 |
| Sa1P | 11.5 ± 0.1 | 7.3 ± 0.4 | 81.2 ± 0.5 |

[a] (peak area of a compound in a nonmethylated mixture – peak area of a nonmethylated compound in a methylated mixture)/peak area of a compound in a nonmethylated mixture × 100 (%)

[b] The number of adducted methoxy group.

[c] The values of percentages are mean ± SD, (n=3).

### 3.3.2 Simultaneous profiling of polar lipids by SFC/MS/MS with methylation

To identify the most effective column for polar lipid profiling by SFC/MS/MS, ODS, C8, diol, and cyano (CN) columns were tested by examining the resolution and peak shape of six PL standards in each case; in particular, peak shape was considered the most important criterion for deciding the optimum column (Figure 3.4). Polar lipids were separated effectively on the YMC Pack-CN column (250 × 4.6 mm ID; 5 μm, Waters), but methylated PC was not detected. YMC Pack-Diol column (250 × 4.6 mm ID; 5 μm, Waters) was ineffective for the detection of methylated PC, and peak tailing was observed for methylated PE. Several ODS columns, too, gave peak tailing for PC. The Inertsil ODS-EP column (250 × 4.6 mm ID; 5 μm, GL Sciences) gave higher resolution than did the other columns but resulted in peak tailing for methylated PE. Inertsil ODS-4 (250 × 4.6 mm ID; 5 μm, GL Sciences), InertSustain C18 (250 × 4.6 mm ID; 5 μm, GL Sciences), and Inertsil C8-4 (250 × 4.6 mm ID; 5 μm, GL Sciences) columns offered good peak shapes for six PLs, although the resolution observed was poor. Thus, the Inertsil ODS-4 column was chosen for polar lipid profiling with methylation.

Next, the modifier ratio, which affects the peak shape and RT, was optimized after examining three modifier gradients: 10-15% (v/v), 15-20% (v/v), and 20-25% (v/v). Polar lipid standards were analyzed by using the Inertsil ODS-4 column with different gradient modifiers for 6 min. When the gradient was 10-15% (v/v), poor peak shapes and low peak intensities were obtained, while a gradient of 15-20% (v/v) gave good results only for PC, PI, and PA. Hence, the modifier gradient that gave the highest peak intensity for the polar lipids, 20-25% (v/v), was chosen.

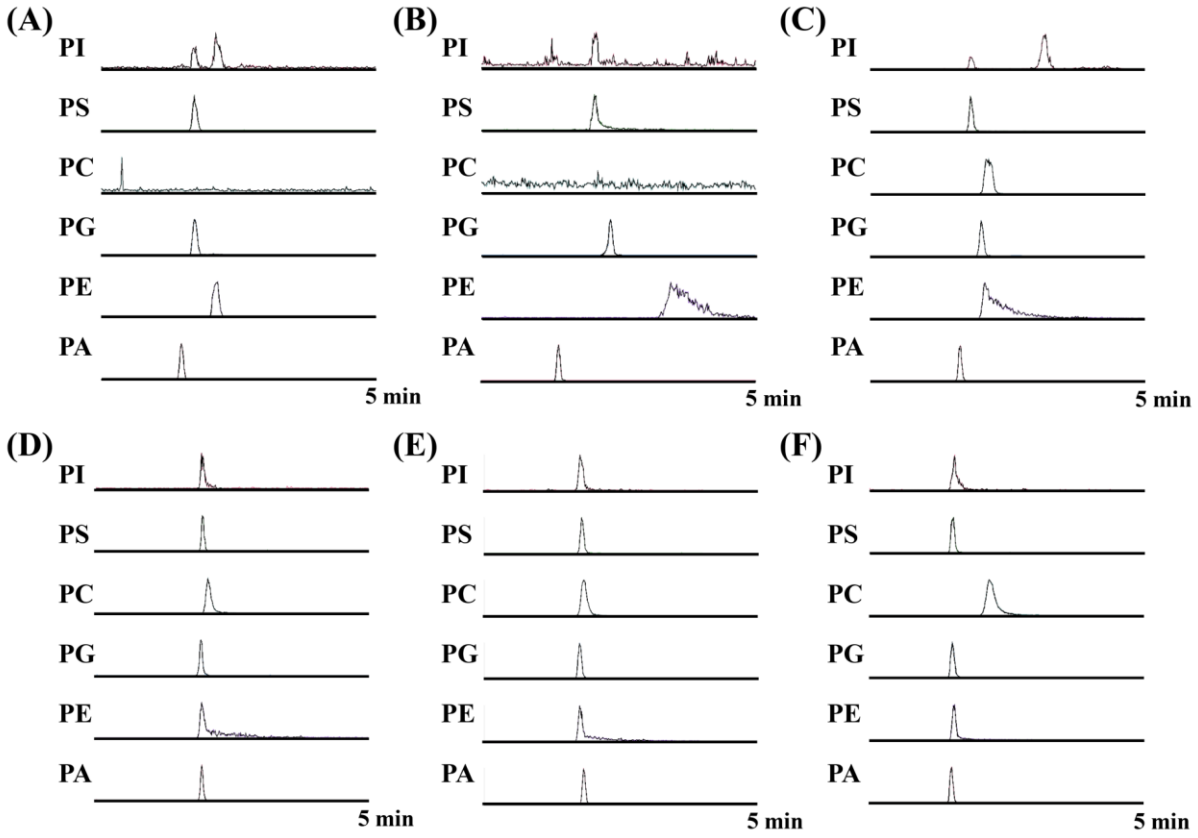


**Figure 3.4** The chromatograms of several columns, tested to examine the resolution and peak shape of six PL standards. Tested columns are as follows: (A) YMC Pack-CN column (250 × 4.6 mm ID; 5 μm, Waters), (B) YMC Pack-Diol column (250 × 4.6 mm ID; 5 μm, Waters), (C) Inertsil ODS-EP column (250 × 4.6 mm ID; 5 μm, GL Sciences), (D) Inertsil ODS-4 column (250 × 4.6 mm ID; 5 μm, GL Sciences), (E) InertSustain C18 column (250 × 4.6 mm ID; 5 μm, GL Sciences), and (F) Inertsil C8-4 column (250 × 4.6 mm ID; 5 μm, GL Sciences).

The results obtained for simultaneous polar lipid profiling with and without methylation were compared to prove the utility of the developed method. Sixteen standards including PC, PE, PS, PG, PI, PA, LPC, LPE, LPS, LPG, LPI, LPA, SM, Cer1P, So1P, and Sa1P were used for the comparison. In the analysis of nonmethylated polar lipids, a hydrogen ion ($H^+$, *m/z* 1.01), an ammonium ion ($NH_4^+$, *m/z* 18.03) and a sodium ion ($Na^+$, *m/z* 22.99) were detected as the adducted ions, both in the positive and negative ion modes. In the positive ion mode,

PG and PA were detected as $[M+NH_4]^+$ ions and LPI was detected as an $[M+Na]^+$ ion. In the negative ion mode, LPG and LPA were detected as $[M–H]^–$ ions. Other polar lipids were detected as $[M+H]^+$ ions in the positive ion mode. The MRM conditions were also optimized to analyze nonmethylated polar lipids (Table 3.3). The column used was the same as that in polar lipid profiling with methylation. Severe peak tailing was observed in the analysis of nonmethylated PS, PA, LPS, LPI, LPA, Cer1P, So1P, and Sa1P, although reasonably good peaks shapes were observed for the other polar lipids. A slightly broad peak was obtained in the case of PI (Figure 3.5A). Methylation helped in suppressing peak tailing considerably in the case of PS, PI, PA, LPS, LPI, LPA, Cer1P, So1P, and Sa1P (Figure 3.5B).

**Table 3.3** Optimized MRM conditions of polar lipid profiling by nonmethylation

| Polar lipids | Ion mode | MRM transitions | CV (V) | MS/MS CE (eV) |
|---|---|---|---|---|
| PC | Positive | [M+H] > 184 | 29 | 30 |
| PE | Positive | [M+H] > [M+H–141] | 23 | 20 |
| PS | Positive | [M+H] > [M+H–185] | 25 | 19 |
| PG | Positive | $[M+NH_4] > [M+NH_4–189]$ | 15 | 16 |
| PI | Positive | [M+H] > [M+H–260] | 22 | 17 |
| PA | Positive | $[M+NH_4] > [M+NH_4–115]$ | 18 | 16 |
| LPC | Positive | [M+H] > 184 | 31 | 26 |
| LPE | Positive | [M+H] > [M+H–141] | 20 | 18 |
| LPS | Positive | [M+H] > [M+H–185] | 22 | 21 |
| LPG | Negative | [M–H] > [M–H–228] | 37 | 25 |
| LPI | Positive | [M+Na] > 283 | 40 | 30 |
| LPA | Negative | [M-H] > 153 | 28 | 22 |
| SM | Positive | [M+H] > 184 | 30 | 26 |
| Cer1P | Positive | [M+H] > 264 | 20 | 30 |
| So1P | Positive | [M+H] > 264 | 20 | 15 |
| Sa1P | Positive | [M+H] > 266 | 24 | 20 |
| Cer | Positive | [M+H] > 264 | 19 | 28 |
| So | Positive | [M+H] > [M+H–18] | 16 | 15 |
| Sa | Positive | [M+H] > [M+H–18] | 25 | 15 |

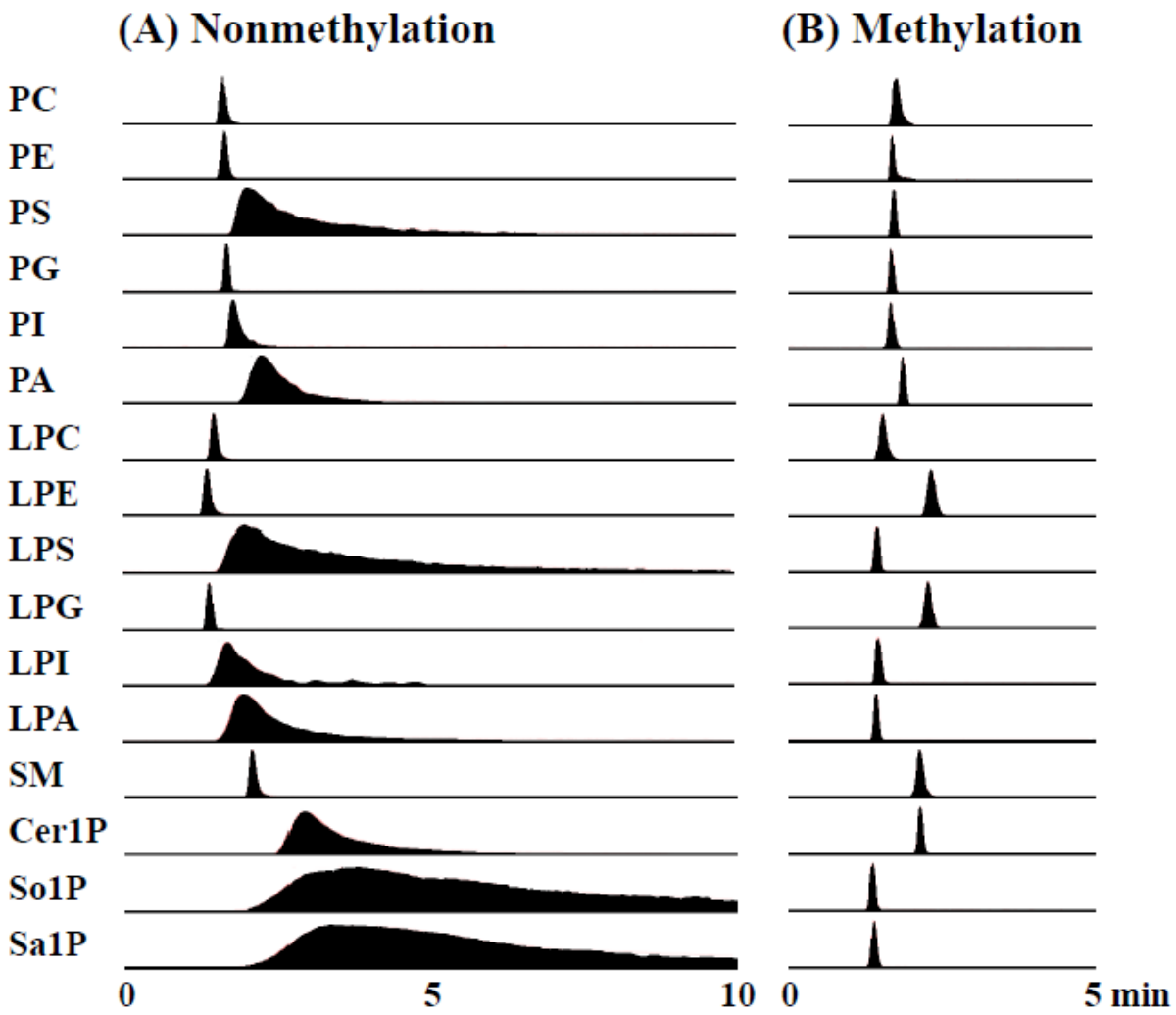


**Figure 3.5** MRM data of the standard PC (12:0-13:0), PE (12:0-13:0), PS (12:0-13:0), PG (12:0-13:0), PI (12:0-13:0), PA (12:0-13:0), LPC (17:0), LPE (14:0), LPS (16:0), LPG (14:0), LPI (18:1), LPA (14:0), SM (d18:1-17:0), Cer1P (d18:1-16:0), So1P (d17:1), and Sa1P (d17:0) by (A) nonmethylation and (B) methylation.

A validation study was performed to estimate the performance of the methylation process for the quantification of polar lipids (Table 3.4). Each polar lipid standard was analyzed with an internal standard, PC (12:0-12:0), and the RSD (%) was calculated to evaluate the precision. The RSDs of the relative (compound/internal standard) retention times and relative peak areas were smaller than 1.3% and 9.4%, indicating high reproducibility. Furthermore, the $R^2$ in each polar lipid analysis was at least 0.9905, indicating high reliability.

The LODs of the nonmethylated PS, PG, PA, LPS, LPG, LPI, LPA, Cer1P, So1P, and Sa1P were 7.5-, 5-, 26.7-, 600-, 6.7-, 116.7-, 500-, 75-, 3000-, and 4500-fold higher than those for the corresponding methylated lipids. However, the LODs of PI, LPC, and LPE were slightly changed: PI, 250 to 200; LPC, 4.5 to 1; and LPE, 30 to 15 fmol. In contrast, the LODs of PC, PE, and SM increased from 0.25 to 2.5, from 2 to 50, and from 1.5 to 9 fmol, respectively.

**Table 3.4** Validation of methylated polar lipid analysis and the LODs comparison with nonmethylated lipid analysis

| Polar | RT | RSD (n=6) (%) | | Correlation | Linear range | LOD (fmol) | |
|---|---|---|---|---|---|---|---|
| lipids | (min) | RT[a] | Peak area[b] | ($R^2$) | (fmol) | Methylated | Nonmethylated |
| PC | 1.64 | 1.0 | 2.0 | 0.9953 | 2.5 – 5000 | 2.5 | 0.25 |
| PE | 1.7 | 1.2 | 4.1 | 0.9956 | 50 – 5000 | 50 | 2 |
| PS | 1.74 | 1.1 | 5.2 | 0.9998 | 20 – 5000 | 20 | 150 |
| PG | 1.69 | 0.6 | 5.6 | 0.9931 | 5 – 5000 | 5 | 25 |
| PI | 1.68 | 1.2 | 7.9 | 0.9924 | 200 – 50000 | 200 | 250 |
| PA | 1.87 | 0.3 | 2.8 | 0.9990 | 7.5 – 2500 | 7.5 | 200 |
| LPC | 1.48 | 1.0 | 5.2 | 0.9911 | 1 – 2500 | 1 | 4.5 |
| LPE | 1.4 | 1.2 | 9.3 | 0.9981 | 15 – 5000 | 15 | 30 |
| LPS | 1.46 | 0.6 | 9.0 | 0.9924 | 5 – 5000 | 5 | 3000 |
| LPG | 1.36 | 1.1 | 3.9 | 0.9911 | 7.5 – 5000 | 7.5 | 50 |
| LPI | 1.49 | 1.3 | 9.0 | 0.9972 | 150 – 25000 | 150 | 17500 |
| LPA | 1.41 | 0.7 | 2.8 | 0.9980 | 2.5 – 5000 | 2.5 | 1250 |
| SM | 2.11 | 0.8 | 6.1 | 0.9926 | 9 – 2500 | 9 | 1.5 |
| Cer1P | 2.1 | 0.7 | 1.5 | 0.9925 | 2 – 5000 | 2 | 150 |
| So1P | 1.38 | 0.6 | 9.4 | 0.9931 | 0.25 – 5000 | 0.25 | 750 |
| Sa1P | 1.39 | 0.6 | 5.4 | 0.9980 | 0.5 – 5000 | 0.5 | 2250 |
| Cer | 2.86 | 0.4 | 4.6 | 0.9942 | 10 – 5000 | - | 10 |
| So | 1.66 | 0.6 | 7.4 | 0.9924 | 5 – 5000 | - | 5 |
| Sa | 1.69 | 1.1 | 6.0 | 0.9922 | 2.5 – 5000 | - | 2.5 |

[a] Relative retention time (compound/internal standard)

[b] Relative peak area (compound/internal standard)

### 3.3.3 Analysis of a biological sample

Next, the lipids extracted from mouse liver were analyzed to check the feasibility of using the developed method for the simultaneous profiling of diverse polar lipids in a biological sample. For the effective extraction of polar lipids, methanol was used as the extraction solvent [130]. PC (12:0-13:0), PE (12:0-13:0), PS (12:0-13:0), PI (12:0-13:0), PG (12:0-13:0), and PA (12:0-13:0) were used as the ISs for the analysis of each PL class. Furthermore, LPC (17:0), SM (d18:0-17:0), Cer (d18:1-17:0), Cer1P (d18:1-12:0), So (d17:1), So1P (d17:1), Sa (d17:0), and Sa1P (d17:0) were the ISs for the analysis of LPL and SL classes. In addition, the following numbers of MRM transitions were used to detect molecular species having different compositions of FAs, on the basis of the number of molecular species expected for each methylated polar lipid (Table 3.5) and nonmethylated polar lipid (Table 3.6): PC, 48; PE, 39; PS, 39; PG, 39; PI, 48; PA, 39; LPC, 25; LPE, 19; LPS, 19; LPG, 19; LPI, 19; LPA, 19; SM, 35; Cer, 35; Cer1P, 35; So, 1; So1P, 1; Sa, 1; and Sa1P, 1.

**Table 3.5** MRM transitions for polar lipid profiling of mouse liver by SFC/MS/MS with methylation

| Fatty acid compositions | PC | | PE | | PS | | PG | | PI | | PA | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cn_Un | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 |
| 42:2 | 884.3 | 198 | - | - | - | - | - | - | 961.6 | 687.4 | - | - |
| 42:3 | 882.3 | 198 | - | - | - | - | - | - | 959.6 | 685.4 | - | - |
| 42:4 | 880.3 | 198 | - | - | - | - | - | - | 957.6 | 683.4 | - | - |
| 42:5 | 878.3 | 198 | - | - | - | - | - | - | 955.6 | 681.4 | - | - |
| 42:6 | 876.3 | 198 | - | - | - | - | - | - | 953.6 | 679.4 | - | - |
| 42:7 | 874.3 | 198 | - | - | - | - | - | - | 951.6 | 677.4 | - | - |
| 42:8 | 872.3 | 198 | - | - | - | - | - | - | 949.6 | 675.4 | - | - |
| 42:9 | 870.3 | 198 | - | - | - | - | - | - | 947.6 | 673.4 | - | - |
| 42:10 | 868.3 | 198 | - | - | - | - | - | - | 945.6 | 671.4 | - | - |
| 40:1 | 858.3 | 198 | 816.6 | 661.3 | 874.6 | 661.3 | 847.3 | 661.3 | 935.6 | 661.3 | 787.5 | 661.3 |
| 40:2 | 856.3 | 198 | 814.6 | 659.3 | 872.6 | 659.3 | 845.3 | 659.3 | 933.6 | 659.3 | 785.5 | 659.3 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 40:3 | 854.3 | 198 | 812.6 | 657.3 | 870.6 | 657.3 | 843.3 | 657.3 | 931.6 | 657.3 | 783.5 | 657.3 |
| 40:4 | 852.3 | 198 | 810.6 | 655.3 | 868.6 | 655.3 | 841.3 | 655.3 | 929.6 | 655.3 | 781.5 | 655.3 |
| 40:5 | 850.4 | 198 | 808.6 | 653.3 | 866.6 | 653.3 | 839.3 | 653.3 | 927.6 | 653.3 | 779.5 | 653.3 |
| 40:6 | 848.3 | 198 | 806.6 | 651.3 | 864.6 | 651.3 | 837.3 | 651.3 | 925.6 | 651.3 | 777.5 | 651.3 |
| 40:7 | 846.3 | 198 | 804.6 | 649.3 | 862.6 | 649.3 | 835.3 | 649.3 | 923.6 | 649.3 | 775.5 | 649.3 |
| 40:8 | 844.3 | 198 | 802.6 | 647.3 | 860.6 | 647.3 | 833.3 | 647.3 | 921.6 | 647.3 | 773.5 | 647.3 |
| 38:0 | 832.3 | 198 | 790.6 | 635.3 | 848.6 | 635.3 | 821.3 | 635.3 | 909.6 | 635.3 | 761.5 | 635.3 |
| 38:1 | 830.3 | 198 | 788.6 | 633.3 | 846.6 | 633.3 | 819.3 | 633.3 | 907.6 | 633.3 | 759.5 | 633.3 |
| 38:2 | 828.3 | 198 | 786.6 | 631.3 | 844.6 | 631.3 | 817.3 | 631.3 | 905.6 | 631.3 | 757.5 | 631.3 |
| 38:3 | 826.3 | 198 | 784.6 | 629.3 | 842.6 | 629.3 | 815.3 | 629.3 | 903.6 | 629.3 | 755.5 | 629.3 |
| 38:4 | 824.3 | 198 | 782.6 | 627.3 | 840.6 | 627.3 | 813.3 | 627.3 | 901.6 | 627.3 | 753.5 | 627.3 |
| 38:5 | 822.3 | 198 | 780.6 | 625.3 | 838.6 | 625.3 | 811.3 | 625.3 | 899.6 | 625.3 | 751.5 | 625.3 |
| 38:6 | 820.3 | 198 | 778.6 | 623.3 | 836.6 | 623.3 | 809.3 | 623.3 | 897.6 | 623.3 | 749.5 | 623.3 |
| 38:7 | 818.3 | 198 | 776.6 | 621.3 | 834.6 | 621.3 | 807.3 | 621.3 | 895.6 | 621.3 | 747.5 | 621.3 |
| 38:8 | 816.3 | 198 | 774.6 | 619.3 | 832.6 | 619.3 | 805.3 | 619.3 | 893.6 | 619.3 | 745.5 | 619.3 |
| 36:0 | 804.3 | 198 | 762.6 | 607.3 | 820.6 | 607.3 | 793.3 | 607.3 | 881.6 | 607.3 | 733.5 | 607.3 |
| 36:1 | 802.3 | 198 | 760.6 | 605.3 | 818.6 | 605.3 | 791.3 | 605.3 | 879.6 | 605.3 | 731.5 | 605.3 |
| 36:2 | 800.3 | 198 | 758.6 | 603.3 | 816.6 | 603.3 | 789.3 | 603.3 | 877.6 | 603.3 | 729.5 | 603.3 |
| 36:3 | 798.3 | 198 | 756.6 | 601.3 | 814.6 | 601.3 | 787.3 | 601.3 | 875.6 | 601.3 | 727.5 | 601.3 |
| 36:4 | 796.3 | 198 | 754.6 | 599.3 | 812.6 | 599.3 | 785.3 | 599.3 | 873.6 | 599.3 | 725.5 | 599.3 |
| 36:5 | 794.3 | 198 | 752.6 | 597.3 | 810.6 | 597.3 | 783.3 | 597.3 | 871.6 | 597.3 | 723.5 | 597.3 |
| 36:6 | 792.3 | 198 | 750.6 | 595.3 | 808.6 | 595.3 | 781.3 | 595.3 | 869.6 | 595.3 | 721.5 | 595.3 |
| 34:0 | 776.3 | 198 | 734.6 | 579.3 | 792.6 | 579.3 | 765.3 | 579.3 | 853.6 | 579.3 | 705.5 | 579.3 |
| 34:1 | 774.3 | 198 | 732.6 | 577.3 | 790.6 | 577.3 | 763.3 | 577.3 | 851.6 | 577.3 | 703.5 | 577.3 |
| 34:2 | 772.3 | 198 | 730.6 | 575.3 | 788.6 | 575.3 | 761.3 | 575.3 | 849.6 | 575.3 | 701.5 | 575.3 |
| 34:3 | 770.3 | 198 | 728.6 | 573.3 | 786.6 | 573.3 | 759.3 | 573.3 | 847.6 | 573.3 | 699.5 | 573.3 |
| 34:4 | 768.3 | 198 | 726.6 | 571.3 | 784.6 | 571.3 | 757.3 | 571.3 | 845.6 | 571.3 | 697.5 | 571.3 |
| 34:5 | 766.3 | 198 | 724.6 | 569.3 | 782.6 | 569.3 | 755.3 | 569.3 | 843.6 | 569.3 | 695.5 | 569.3 |
| 34:6 | 764.3 | 198 | 722.6 | 567.3 | 780.6 | 567.3 | 753.3 | 567.3 | 841.6 | 567.3 | 693.5 | 567.3 |
| 32:0 | 748.3 | 198 | 706.6 | 551.3 | 764.6 | 551.3 | 737.3 | 551.3 | 825.6 | 551.3 | 677.5 | 551.3 |
| 32:1 | 746.3 | 198 | 704.6 | 549.3 | 762.6 | 549.3 | 735.3 | 549.3 | 823.6 | 549.3 | 675.5 | 549.3 |
| 32:2 | 744.3 | 198 | 702.6 | 547.3 | 760.6 | 547.3 | 733.3 | 547.3 | 821.6 | 547.3 | 673.5 | 547.3 |
| 32:3 | 742.3 | 198 | 700.6 | 545.3 | 758.6 | 545.3 | 731.3 | 545.3 | 819.6 | 545.3 | 671.5 | 545.3 |
| 32:4 | 740.3 | 198 | 698.6 | 543.3 | 756.6 | 543.3 | 729.3 | 543.3 | 817.6 | 543.3 | 669.5 | 543.3 |
| 30:0 | 720.3 | 198 | 678.6 | 523.3 | 736.6 | 523.3 | 709.3 | 523.3 | 797.6 | 523.3 | 649.5 | 523.3 |
| 30:1 | 718.3 | 198 | 676.6 | 521.3 | 734.6 | 521.3 | 707.3 | 521.3 | 795.6 | 521.3 | 647.5 | 521.3 |
| 30:2 | 716.3 | 198 | 674.6 | 519.3 | 732.6 | 519.3 | 705.3 | 519.3 | 793.6 | 519.3 | 645.5 | 519.3 |

**Table 3.5** (Continued)

| | LPC | | LPE | | LPS | | LPG | | LPI | | LPA | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cn_Un | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 |
| 24:2 | 618.4 | 198 | - | - | - | - | - | - | - | - | - | - |
| 24:3 | 616.4 | 198 | - | - | - | - | - | - | - | - | - | - |
| 24:4 | 614.4 | 198 | - | - | - | - | - | - | - | - | - | - |
| 24:5 | 612.4 | 198 | - | - | - | - | - | - | - | - | - | - |
| 24:6 | 610.4 | 198 | - | - | - | - | - | - | - | - | - | - |
| 24:7 | 608.4 | 198 | - | - | - | - | - | - | - | - | - | - |
| 22:1 | 592.4 | 198 | 550.3 | 395.2 | 608.3 | 395.2 | 581.3 | 395.2 | 691.3 | 297 | 521.3 | 395.2 |
| 22:2 | 590.4 | 198 | 548.3 | 393.2 | 606.3 | 393.2 | 579.3 | 393.2 | 689.3 | 297 | 519.3 | 393.2 |
| 22:3 | 588.4 | 198 | 546.3 | 391.2 | 604.3 | 391.2 | 577.3 | 391.2 | 687.3 | 297 | 517.3 | 391.2 |
| 22:4 | 586.4 | 198 | 544.3 | 389.2 | 602.3 | 389.2 | 575.3 | 389.2 | 685.3 | 297 | 515.3 | 389.2 |
| 22:5 | 584.4 | 198 | 542.3 | 387.2 | 600.3 | 387.2 | 573.3 | 387.2 | 683.3 | 297 | 513.3 | 387.2 |
| 22:6 | 582.4 | 198 | 540.3 | 385.2 | 598.3 | 385.2 | 571.3 | 385.2 | 681.3 | 297 | 511.3 | 385.2 |
| 20:0 | 566.4 | 198 | 524.3 | 369.2 | 582.3 | 369.2 | 555.3 | 369.2 | 665.3 | 297 | 495.3 | 369.2 |
| 20:1 | 564.4 | 198 | 522.3 | 367.2 | 580.3 | 367.2 | 553.3 | 367.2 | 663.3 | 297 | 493.3 | 367.2 |
| 20:2 | 562.4 | 198 | 520.3 | 365.2 | 578.3 | 365.2 | 551.3 | 365.2 | 661.3 | 297 | 491.3 | 365.2 |
| 20:3 | 560.4 | 198 | 518.3 | 363.2 | 576.3 | 363.2 | 549.3 | 363.2 | 659.3 | 297 | 489.3 | 363.2 |
| 20:4 | 558.4 | 198 | 516.3 | 361.2 | 574.3 | 361.2 | 547.3 | 361.2 | 657.3 | 297 | 487.3 | 361.2 |
| 18:0 | 538.4 | 198 | 496.3 | 341.2 | 554.3 | 341.2 | 527.3 | 341.2 | 637.3 | 297 | 467.3 | 341.2 |
| 18:1 | 536.4 | 198 | 494.3 | 339.2 | 552.3 | 339.2 | 525.3 | 339.2 | 635.3 | 297 | 465.3 | 339.2 |
| 18:2 | 534.4 | 198 | 492.3 | 337.2 | 550.3 | 337.2 | 523.3 | 337.2 | 633.3 | 297 | 463.3 | 337.2 |
| 18:3 | 532.4 | 198 | 490.3 | 335.2 | 548.3 | 335.2 | 521.3 | 335.2 | 631.3 | 297 | 461.3 | 335.2 |
| 16:0 | 510.4 | 198 | 468.3 | 313.2 | 526.3 | 313.2 | 499.3 | 313.2 | 609.3 | 297 | 439.3 | 313.2 |
| 16:1 | 508.4 | 198 | 466.3 | 311.2 | 524.3 | 311.2 | 497.3 | 311.2 | 607.3 | 297 | 437.3 | 311.2 |
| 16:2 | 506.4 | 198 | 464.3 | 309.2 | 522.3 | 309.2 | 495.3 | 309.2 | 605.3 | 297 | 435.3 | 309.2 |
| 14:0 | 482.4 | 198 | 440.3 | 285.2 | 498.3 | 285.2 | 471.3 | 285.2 | 581.3 | 297 | 411.3 | 285.2 |

**Table 3.5** (Continued)

| | SM | | Cer1P | | So1P | | | Sa1P | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Cn_Un | Q1 | Q3 | Q1 | Q3 | Cn_Un | Q1 | Q3 | Cn_Un | Q1 | Q3 |
| d18:1-28:0 | 886.6 | 198 | 814.5 | 264 | d18:1 | 408 | 264 | d18:0 | 410 | 266 |
| d18:1-28:1 | 884.6 | 198 | 812.5 | 264 | | | | | | |
| d18:1-28:2 | 882.6 | 198 | 810.5 | 264 | | | | | | |
| d18:1-26:0 | 858.6 | 198 | 786.5 | 264 | | | | | | |
| d18:1-26:1 | 856.6 | 198 | 784.5 | 264 | | | | | | |
| d18:1-26:2 | 854.6 | 198 | 782.5 | 264 | | | | | | |
| d18:1-26:3 | 852.6 | 198 | 780.5 | 264 | | | | | | |
| d18:1-26:4 | 850.6 | 198 | 778.5 | 264 | | | | | | |
| d18:1-26:5 | 848.6 | 198 | 776.5 | 264 | | | | | | |
| d18:1-26:6 | 846.6 | 198 | 774.5 | 264 | | | | | | |
| d18:1-24:0 | 830.6 | 198 | 758.5 | 264 | | | | | | |
| d18:1-24:1 | 828.6 | 198 | 756.5 | 264 | | | | | | |
| d18:1-24:2 | 826.6 | 198 | 754.5 | 264 | | | | | | |
| d18:1-24:3 | 824.6 | 198 | 752.5 | 264 | | | | | | |
| d18:1-24:4 | 822.6 | 198 | 750.5 | 264 | | | | | | |
| d18:1-24:5 | 820.6 | 198 | 748.5 | 264 | | | | | | |
| d18:1-24:6 | 818.6 | 198 | 746.5 | 264 | | | | | | |
| d18:1-22:0 | 802.6 | 198 | 730.5 | 264 | | | | | | |
| d18:1-22:1 | 800.6 | 198 | 728.5 | 264 | | | | | | |
| d18:1-22:2 | 798.6 | 198 | 726.5 | 264 | | | | | | |
| d18:1-22:3 | 796.6 | 198 | 724.5 | 264 | | | | | | |
| d18:1-22:4 | 794.6 | 198 | 722.5 | 264 | | | | | | |
| d18:1-22:5 | 792.6 | 198 | 720.5 | 264 | | | | | | |
| d18:1-22:6 | 790.6 | 198 | 718.5 | 264 | | | | | | |
| d18:1-20:0 | 774.6 | 198 | 702.5 | 264 | | | | | | |
| d18:1-20:1 | 772.6 | 198 | 700.5 | 264 | | | | | | |
| d18:1-20:2 | 770.6 | 198 | 698.5 | 264 | | | | | | |
| d18:1-20:3 | 768.6 | 198 | 696.5 | 264 | | | | | | |
| d18:1-20:4 | 766.6 | 198 | 694.5 | 264 | | | | | | |
| d18:1-18:0 | 746.6 | 198 | 674.5 | 264 | | | | | | |
| d18:1-18:1 | 744.6 | 198 | 672.5 | 264 | | | | | | |
| d18:1-18:2 | 742.6 | 198 | 670.5 | 264 | | | | | | |
| d18:1-16:0 | 718.6 | 198 | 646.5 | 264 | | | | | | |
| d18:1-16:1 | 716.6 | 198 | 644.5 | 264 | | | | | | |
| d18:1-14:0 | 690.6 | 198 | 618.5 | 264 | | | | | | |

**Table 3.6** MRM transitions for polar lipid profiling of mouse liver by SFC/MS/MS with nonmethylation.

| Fatty acid compositions | PC | | PE | | PS | | PG | | PI | | PA | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cn_Un | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 |
| 42:2 | 870.5 | 184 | - | - | - | - | - | - | 947.5 | 687.4 | - | - |
| 42:3 | 868.5 | 184 | - | - | - | - | - | - | 945.5 | 685.4 | - | - |
| 42:4 | 866.5 | 184 | - | - | - | - | - | - | 943.5 | 683.4 | - | - |
| 42:5 | 864.5 | 184 | - | - | - | - | - | - | 941.5 | 681.4 | - | - |
| 42:6 | 862.5 | 184 | - | - | - | - | - | - | 939.5 | 679.4 | - | - |
| 42:7 | 860.5 | 184 | - | - | - | - | - | - | 937.5 | 677.4 | - | - |
| 42:8 | 858.5 | 184 | - | - | - | - | - | - | 935.5 | 675.4 | - | - |
| 42:9 | 856.5 | 184 | - | - | - | - | - | - | 933.5 | 673.4 | - | - |
| 42:10 | 854.5 | 184 | - | - | - | - | - | - | 931.5 | 671.4 | - | - |
| 40:1 | 844.5 | 184 | 802.4 | 661.3 | 846.4 | 661.3 | 850.4 | 661.3 | 921.5 | 661.3 | 776.3 | 661.3 |
| 40:2 | 842.5 | 184 | 800.4 | 659.3 | 844.4 | 659.3 | 848.4 | 659.3 | 919.5 | 659.3 | 774.3 | 659.3 |
| 40:3 | 840.5 | 184 | 798.4 | 657.3 | 842.4 | 657.3 | 846.4 | 657.3 | 917.5 | 657.3 | 772.3 | 657.3 |
| 40:4 | 838.5 | 184 | 796.4 | 655.3 | 840.4 | 655.3 | 844.4 | 655.3 | 915.5 | 655.3 | 770.3 | 655.3 |
| 40:5 | 836.5 | 184 | 794.4 | 653.3 | 838.4 | 653.3 | 842.4 | 653.3 | 913.5 | 653.3 | 768.3 | 653.3 |
| 40:6 | 834.5 | 184 | 792.4 | 651.3 | 836.4 | 651.3 | 840.4 | 651.3 | 911.5 | 651.3 | 766.3 | 651.3 |
| 40:7 | 832.5 | 184 | 790.4 | 649.3 | 834.4 | 649.3 | 838.4 | 649.3 | 909.5 | 649.3 | 764.3 | 649.3 |
| 40:8 | 830.5 | 184 | 788.4 | 647.3 | 832.4 | 647.3 | 836.4 | 647.3 | 907.5 | 647.3 | 762.3 | 647.3 |
| 38:0 | 818.5 | 184 | 776.4 | 635.3 | 820.4 | 635.3 | 824.4 | 635.3 | 895.5 | 635.3 | 750.3 | 635.3 |
| 38:1 | 816.5 | 184 | 774.4 | 633.3 | 818.4 | 633.3 | 822.4 | 633.3 | 893.5 | 633.3 | 748.3 | 633.3 |
| 38:2 | 814.5 | 184 | 772.4 | 631.3 | 816.4 | 631.3 | 820.4 | 631.3 | 891.5 | 631.3 | 746.3 | 631.3 |
| 38:3 | 812.5 | 184 | 770.4 | 629.3 | 814.4 | 629.3 | 818.4 | 629.3 | 889.5 | 629.3 | 744.3 | 629.3 |
| 38:4 | 810.5 | 184 | 768.4 | 627.3 | 812.4 | 627.3 | 816.4 | 627.3 | 887.5 | 627.3 | 742.3 | 627.3 |
| 38:5 | 808.5 | 184 | 766.4 | 625.3 | 810.4 | 625.3 | 814.4 | 625.3 | 885.5 | 625.3 | 740.3 | 625.3 |
| 38:6 | 806.5 | 184 | 764.4 | 623.3 | 808.4 | 623.3 | 812.4 | 623.3 | 883.5 | 623.3 | 738.3 | 623.3 |
| 38:7 | 804.5 | 184 | 762.4 | 621.3 | 806.4 | 621.3 | 810.4 | 621.3 | 881.5 | 621.3 | 736.3 | 621.3 |
| 38:8 | 802.5 | 184 | 760.4 | 619.3 | 804.4 | 619.3 | 808.4 | 619.3 | 879.5 | 619.3 | 734.3 | 619.3 |
| 36:0 | 790.5 | 184 | 748.4 | 607.3 | 792.4 | 607.3 | 796.4 | 607.3 | 867.5 | 607.3 | 722.3 | 607.3 |
| 36:1 | 788.5 | 184 | 746.4 | 605.3 | 790.4 | 605.3 | 794.4 | 605.3 | 865.5 | 605.3 | 720.3 | 605.3 |
| 36:2 | 786.5 | 184 | 744.4 | 603.3 | 788.4 | 603.3 | 792.4 | 603.3 | 863.5 | 603.3 | 718.3 | 603.3 |
| 36:3 | 784.5 | 184 | 742.4 | 601.3 | 786.4 | 601.3 | 790.4 | 601.3 | 861.5 | 601.3 | 716.3 | 601.3 |
| 36:4 | 782.5 | 184 | 740.4 | 599.3 | 784.4 | 599.3 | 788.4 | 599.3 | 859.5 | 599.3 | 714.3 | 599.3 |
| 36:5 | 780.5 | 184 | 738.4 | 597.3 | 782.4 | 597.3 | 786.4 | 597.3 | 857.5 | 597.3 | 712.3 | 597.3 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 36:6 | 778.5 | 184 | 736.4 | 595.3 | 780.4 | 595.3 | 784.4 | 595.3 | 855.5 | 595.3 | 710.3 | 595.3 |
| 34:0 | 762.5 | 184 | 720.4 | 579.3 | 764.4 | 579.3 | 768.4 | 579.3 | 839.5 | 579.3 | 694.3 | 579.3 |
| 34:1 | 760.5 | 184 | 718.4 | 577.3 | 762.4 | 577.3 | 766.4 | 577.3 | 837.5 | 577.3 | 692.3 | 577.3 |
| 34:2 | 758.5 | 184 | 716.4 | 575.3 | 760.4 | 575.3 | 764.4 | 575.3 | 835.5 | 575.3 | 690.3 | 575.3 |
| 34:3 | 756.5 | 184 | 714.4 | 573.3 | 758.4 | 573.3 | 762.4 | 573.3 | 833.5 | 573.3 | 688.3 | 573.3 |
| 34:4 | 754.5 | 184 | 712.4 | 571.3 | 756.4 | 571.3 | 760.4 | 571.3 | 831.5 | 571.3 | 686.3 | 571.3 |
| 34:5 | 752.5 | 184 | 710.4 | 569.3 | 754.4 | 569.3 | 758.4 | 569.3 | 829.5 | 569.3 | 684.3 | 569.3 |
| 34:6 | 750.5 | 184 | 708.4 | 567.3 | 752.4 | 567.3 | 756.4 | 567.3 | 827.5 | 567.3 | 682.3 | 567.3 |
| 32:0 | 734.5 | 184 | 692.4 | 551.3 | 736.4 | 551.3 | 740.4 | 551.3 | 811.5 | 551.3 | 666.3 | 551.3 |
| 32:1 | 732.5 | 184 | 690.4 | 549.3 | 734.4 | 549.3 | 738.4 | 549.3 | 809.5 | 549.3 | 664.3 | 549.3 |
| 32:2 | 730.5 | 184 | 688.4 | 547.3 | 732.4 | 547.3 | 736.4 | 547.3 | 807.5 | 547.3 | 662.3 | 547.3 |
| 32:3 | 728.5 | 184 | 686.4 | 545.3 | 730.4 | 545.3 | 734.4 | 545.3 | 805.5 | 545.3 | 660.3 | 545.3 |
| 32:4 | 726.5 | 184 | 684.4 | 543.3 | 728.4 | 543.3 | 732.4 | 543.3 | 803.5 | 543.3 | 658.3 | 543.3 |
| 30:0 | 706.5 | 184 | 664.4 | 523.3 | 708.4 | 523.3 | 712.4 | 523.3 | 783.5 | 523.3 | 638.3 | 523.3 |
| 30:1 | 704.5 | 184 | 662.4 | 521.3 | 706.4 | 521.3 | 710.4 | 521.3 | 781.5 | 521.3 | 636.3 | 521.3 |
| 30:2 | 702.5 | 184 | 660.4 | 519.3 | 704.4 | 519.3 | 708.4 | 519.3 | 779.5 | 519.3 | 634.3 | 519.3 |

**Table 3.6** (Continued)

| | LPC | | LPE | | LPS | | LPG | | LPI | | LPA | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cn_Un | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 |
| 24:2 | 604.1 | 184 | - | - | - | - | - | - | - | - | - | - |
| 24:3 | 602.1 | 184 | - | - | - | - | - | - | - | - | - | - |
| 24:4 | 600.1 | 184 | - | - | - | - | - | - | - | - | - | - |
| 24:5 | 598.1 | 184 | - | - | - | - | - | - | - | - | - | - |
| 24:6 | 596.1 | 184 | - | - | - | - | - | - | - | - | - | - |
| 24:7 | 594.1 | 184 | - | - | - | - | - | - | - | - | - | - |
| 22:1 | 578.1 | 184 | 536.3 | 395.2 | 580.3 | 395.2 | 565.1 | 395.2 | 677.1 | 283 | 491.1 | 153 |
| 22:2 | 576.1 | 184 | 534.3 | 393.2 | 578.3 | 393.2 | 563.1 | 393.2 | 675.1 | 283 | 489.1 | 153 |
| 22:3 | 574.1 | 184 | 532.3 | 391.2 | 576.3 | 391.2 | 561.1 | 391.2 | 673.1 | 283 | 487.1 | 153 |
| 22:4 | 572.1 | 184 | 530.3 | 389.2 | 574.3 | 389.2 | 559.1 | 389.2 | 671.1 | 283 | 485.1 | 153 |
| 22:5 | 570.1 | 184 | 528.3 | 387.2 | 572.3 | 387.2 | 557.1 | 387.2 | 669.1 | 283 | 483.1 | 153 |
| 22:6 | 568.1 | 184 | 526.3 | 385.2 | 570.3 | 385.2 | 555.1 | 385.2 | 667.1 | 283 | 481.1 | 153 |
| 20:0 | 552.1 | 184 | 510.3 | 369.2 | 554.3 | 369.2 | 539.1 | 369.2 | 651.1 | 283 | 465.1 | 153 |
| 20:1 | 550.1 | 184 | 508.3 | 367.2 | 552.3 | 367.2 | 537.1 | 367.2 | 649.1 | 283 | 463.1 | 153 |
| 20:2 | 548.1 | 184 | 506.3 | 365.2 | 550.3 | 365.2 | 535.1 | 365.2 | 647.1 | 283 | 461.1 | 153 |
| 20:3 | 546.1 | 184 | 504.3 | 363.2 | 548.3 | 363.2 | 533.1 | 363.2 | 645.1 | 283 | 459.1 | 153 |
| 20:4 | 544.1 | 184 | 502.3 | 361.2 | 546.3 | 361.2 | 531.1 | 361.2 | 643.1 | 283 | 457.1 | 153 |
| 18:0 | 524.1 | 184 | 482.3 | 341.2 | 526.3 | 341.2 | 511.1 | 341.2 | 623.1 | 283 | 437.1 | 153 |
| 18:1 | 522.1 | 184 | 480.3 | 339.2 | 524.3 | 339.2 | 509.1 | 339.2 | 621.1 | 283 | 435.1 | 153 |
| 18:2 | 520.1 | 184 | 478.3 | 337.2 | 522.3 | 337.2 | 507.1 | 337.2 | 619.1 | 283 | 433.1 | 153 |
| 18:3 | 518.1 | 184 | 476.3 | 335.2 | 520.3 | 335.2 | 505.1 | 335.2 | 617.1 | 283 | 431.1 | 153 |
| 16:0 | 496.1 | 184 | 454.3 | 313.2 | 498.3 | 313.2 | 483.1 | 313.2 | 595.1 | 283 | 409.1 | 153 |
| 16:1 | 494.1 | 184 | 452.3 | 311.2 | 496.3 | 311.2 | 481.1 | 311.2 | 593.1 | 283 | 407.1 | 153 |
| 16:2 | 492.1 | 184 | 450.3 | 309.2 | 494.3 | 309.2 | 479.1 | 309.2 | 591.1 | 283 | 405.1 | 153 |
| 14:0 | 468.1 | 184 | 426.3 | 285.2 | 470.3 | 285.2 | 455.1 | 285.2 | 567.1 | 283 | 381.1 | 153 |

**Table 3.6** (Continued)

| | SM | | Cer | | Cer1P | | | So | |
|---|---|---|---|---|---|---|---|---|---|
| Cn_Un | Q1 | Q3 | Q1 | Q3 | Q1 | Q3 | Cn_Un | Q1 | Q3 |
| d18:1-28:0 | 871.3 | 184 | 706.5 | 264 | 786.5 | 264 | d18:1 | 300 | 264 |
| d18:1-28:1 | 869.3 | 184 | 704.5 | 264 | 784.5 | 264 | So1P | | |
| d18:1-28:2 | 867.3 | 184 | 702.5 | 264 | 782.5 | 264 | Cn_Un | Q1 | Q3 |
| d18:1-26:0 | 843.3 | 184 | 678.5 | 264 | 758.5 | 264 | d18:1 | 380 | 264 |
| d18:1-26:1 | 841.3 | 184 | 676.5 | 264 | 756.5 | 264 | Sa | | |
| d18:1-26:2 | 839.3 | 184 | 674.5 | 264 | 754.5 | 264 | Cn_Un | Q1 | Q3 |
| d18:1-26:3 | 837.3 | 184 | 672.5 | 264 | 752.5 | 264 | d18:0 | 302 | 266 |
| d18:1-26:4 | 835.3 | 184 | 670.5 | 264 | 750.5 | 264 | Sa1P | | |
| d18:1-26:5 | 833.3 | 184 | 668.5 | 264 | 748.5 | 264 | Cn_Un | Q1 | Q3 |
| d18:1-26:6 | 831.3 | 184 | 666.5 | 264 | 746.5 | 264 | d18:0 | 382 | 266 |
| d18:1-24:0 | 815.3 | 184 | 650.5 | 264 | 730.5 | 264 | | | |
| d18:1-24:1 | 813.3 | 184 | 648.5 | 264 | 728.5 | 264 | | | |
| d18:1-24:2 | 811.3 | 184 | 646.5 | 264 | 726.5 | 264 | | | |
| d18:1-24:3 | 809.3 | 184 | 644.5 | 264 | 724.5 | 264 | | | |
| d18:1-24:4 | 807.3 | 184 | 642.5 | 264 | 722.5 | 264 | | | |
| d18:1-24:5 | 805.3 | 184 | 640.5 | 264 | 720.5 | 264 | | | |
| d18:1-24:6 | 803.3 | 184 | 638.5 | 264 | 718.5 | 264 | | | |
| d18:1-22:0 | 787.3 | 184 | 622.5 | 264 | 702.5 | 264 | | | |
| d18:1-22:1 | 785.3 | 184 | 620.5 | 264 | 700.5 | 264 | | | |
| d18:1-22:2 | 783.3 | 184 | 618.5 | 264 | 698.5 | 264 | | | |
| d18:1-22:3 | 781.3 | 184 | 616.5 | 264 | 696.5 | 264 | | | |
| d18:1-22:4 | 779.3 | 184 | 614.5 | 264 | 694.5 | 264 | | | |
| d18:1-22:5 | 777.3 | 184 | 612.5 | 264 | 692.5 | 264 | | | |
| d18:1-22:6 | 775.3 | 184 | 610.5 | 264 | 690.5 | 264 | | | |
| d18:1-20:0 | 759.3 | 184 | 594.5 | 264 | 674.5 | 264 | | | |
| d18:1-20:1 | 757.3 | 184 | 592.5 | 264 | 672.5 | 264 | | | |
| d18:1-20:2 | 755.3 | 184 | 590.5 | 264 | 670.5 | 264 | | | |
| d18:1-20:3 | 753.3 | 184 | 588.5 | 264 | 668.5 | 264 | | | |
| d18:1-20:4 | 751.3 | 184 | 586.5 | 264 | 666.5 | 264 | | | |
| d18:1-18:0 | 731.3 | 184 | 566.5 | 264 | 646.5 | 264 | | | |
| d18:1-18:1 | 729.3 | 184 | 564.5 | 264 | 644.5 | 264 | | | |
| d18:1-18:2 | 727.3 | 184 | 562.5 | 264 | 642.5 | 264 | | | |
| d18:1-16:0 | 703.3 | 184 | 538.5 | 264 | 618.5 | 264 | | | |
| d18:1-16:1 | 701.3 | 184 | 536.5 | 264 | 616.5 | 264 | | | |
| d18:1-14:0 | 675.3 | 184 | 510.5 | 264 | 590.5 | 264 | | | |

In this study, the relative area (peak area/total peak area × 100 (%)) of various polar lipid species was characterized for the detailed phenotype of mouse liver. In order to obtain the reliable quantification, lipid extracts were diluted in three different ratios (1:1, 1:2, and 1:10) and analyzed three times (n=3). Furthermore, the results of polar lipid profiling with and without methylation were compared. Upon methylation, 40 PCs, 23 PEs, 16 PSs, 4 PGs, 12 PIs, 33 PAs, 17 LPCs, 16 LPEs, 14 LPSs, 10 LPGs, 6 LPIs, 22 LPAs, 34 SMs, 18 Cer1Ps, So1P, and Sa1P were successfully analyzed in 6 min. Comparing to nonmethylation analysis, 4 PSs, 24 PAs, 3 LPEs, 11 LPSs, 6 LPGs, 4 LPIs, 13 LPAs, 7 SMs, 11 Cer1Ps, So1P, and Sa1P were additionally analyzed (Table 3.7).

However, the nonmethylation process was better for the analysis of PC, PE, PG, PI, and LPC (Table 3.8). According to the three dilution ratios, each species was quantified in reasonable proportion. Although the analysis of minor lipid species showed relatively low repeatability owing to their low abundance in a mixture, their quantifications were also reasonably accurate. By the nonmethylation process, several polar lipids such as 18 Cers, So, and Sa in mouse liver were analyzed (Table 3.9). Although these three lipid classes were not methylated, they were also analyzed successfully even in a methylated mixture.

**Table 3.7** Molecular species of PS, PA, LPE, LPS, LPG, LPI, LPA, SM, Cer1P, So1P, and Sa1P analyzed in mouse liver by SFC/MS/MS with methylation and the comparison with nonmethylation

| Polar lipid | Cn_ Un | Methylation | | | | | Nonmethylation | |
|---|---|---|---|---|---|---|---|---|
| | | RT (min) | Peak area (1:10)[a] | Peak area (1:2) | Peak area (1:1) | Relative area (%) | RT (min) | Detection |
| PS | 40:4 | 3.12 | 20 ± 8[b] | 78 ± 17 | 237 ± 46 | 1.5 ± 0.3[c] | | N.D. |
| | 40:5 | 3 | 152 ± 31 | 600 ± 130 | 1340 ± 240 | 8.4 ± 1.5 | 3.02 | |
| | 40:6 | 2.95 | 319 ± 67 | 1780 ± 250 | 3400 ± 490 | 21.2 ± 3 | 2.87 | |
| | 40:7 | 2.81 | 26 ± 10 | 154 ± 18 | 398 ± 47 | 2.5 ± 0.3 | 2.65 | |
| | 38:2 | 3.05 | 8.8 ± 2 | 45 ± 12 | 89 ± 17 | 0.6 ± 0.1 | | N.D. |
| | 38:3 | 2.94 | 99 ± 34.1 | 548 ± 90 | 1090 ± 110 | 6.8 ± 0.7 | 2.88 | |
| | 38:4 | 2.87 | 309.2 ± 78.2 | 1550 ± 190 | 3710 ± 350 | 23.1 ± 2.2 | 2.78 | |
| | 38:5 | 2.73 | 122 ± 40 | 630 ± 100 | 1300 ± 200 | 8.3 ± 1.3 | 2.71 | |
| | 38:6 | 2.72 | 82 ± 30 | 529 ± 92 | 1360 ± 200 | 8.5 ± 1.2 | 2.6 | |
| | 36:1 | 2.97 | 50 ± 16 | 179 ± 10 | 505 ± 45 | 3.1 ± 0.3 | 3.08 | |
| | 36:2 | 2.86 | 94 ± 37 | 394 ± 78 | 936 ± 137 | 5.8 ± 0.9 | 2.83 | |
| | 36:3 | 2.69 | 38 ± 14 | 157 ± 32 | 356 ± 44 | 2.2 ± 0.3 | 2.65 | |
| | 36:4 | 2.61 | 75 ± 22 | 299 ± 59 | 692 ± 114 | 4.3 ± 0.7 | 2.58 | |
| | 36:5 | 2.5 | 14.6 ± 4.5 | 80 ± 21 | 161 ± 24 | 1 ± 0.1 | 2.41 | |
| | 34:1 | 2.7 | 13.5 ± 5.4 | 62 ± 11 | 150 ± 23 | 0.9 ± 0.1 | | N.D. |
| | 34:2 | 2.64 | | 46 ± 12 | 287 ± 52 | 1.8 ± 0.3 | | N.D. |
| PA | 40:2 | 4.04 | | | 17.9 ± 9.5 | 0.08 ± 0.04 | | N.D. |
| | 40:3 | 3.6 | | | 21.8 ± 10.2 | 0.1 ± 0.05 | | N.D. |
| | 40:4 | 3.5 | 18.3 ± 11.9 | 87 ± 19 | 128 ± 13 | 0.57 ± 0.06 | | N.D. |
| | 40:5 | 3.33 | 94 ± 34 | 677 ± 43 | 1010 ± 45 | 4.5 ± 0.2 | 3.16 | |
| | 40:6 | 3.26 | 384 ± 147 | 1650 ± 340 | 2800 ± 400 | 12.3 ± 1.8 | 3.09 | |
| | 40:7 | 3.19 | 73 ± 26 | 327 ± 74 | 667 ± 80 | 3 ± 0.4 | | N.D. |
| | 40:8 | 3.05 | 25 ± 15.5 | 86 ± 21 | 126 ± 12 | 0.6 ± 0.05 | | N.D. |
| | 38:1 | 3.69 | | 15.5 ± 5.2 | 51 ± 12 | 0.22 ± 0.05 | | N.D. |
| | 38:2 | 3.33 | | 114 ± 26 | 223 ± 15 | 1 ± 0.06 | | N.D. |
| | 38:3 | 3.29 | 110 ± 66 | 790 ± 210 | 1490 ± 260 | 6.6 ± 1.1 | | N.D. |
| | 38:4 | 3.17 | 385 ± 107 | 2030 ± 550 | 4850 ± 760 | 22 ± 3.4 | | N.D. |
| | 38:5 | 3.03 | 186 ± 39 | 1110 ± 310 | 1990 ± 260 | 8.8 ± 1.2 | 3.14 | |
| | 38:6 | 2.93 | 151 ± 32 | 800 ± 130 | 1660 ± 210 | 7.4 ± 0.9 | 3.08 | |
| | 38:7 | 2.83 | 13.7 ± 12.5 | 64 ± 29 | 115 ± 47 | 0.5 ± 0.2 | | N.D. |
| | 38:8 | 2.69 | | | 12.7 ± 10.7 | 0.06 ± 0.05 | | N.D. |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 36:1 | 3.33 | 77 ± 26 | 480 ± 140 | 1130 ± 160 | 5 ± 0.7 | | N.D. |
| | 36:2 | 3.16 | 177 ± 58 | 638 ± 96 | 1030 ± 130 | 4.6 ± 0.6 | 3.26 | |
| | 36:3 | 2.94 | 90 ± 47 | 456 ± 192 | 738 ± 86 | 3.3 ± 0.4 | 3.13 | |
| | 36:4 | 2.86 | 105 ± 18 | 627 ± 104 | 1060 ± 150 | 4.7 ± 0.6 | 2.99 | |
| | 36:5 | 2.79 | 36 ± 27 | 215 ± 109 | 318 ± 74 | 1.4 ± 0.3 | | N.D. |
| | 36:6 | 2.6 | | | 35.1 ± 26 | 0.16 ± 0.12 | | N.D. |
| | 34:0 | 3.01 | | 96 ± 39 | 188 ± 32 | 0.8 ± 0.1 | | N.D. |
| | 34:1 | 2.94 | 95 ± 16 | 687 ± 131 | 1020 ± 90 | 4.5 ± 0.4 | | N.D. |
| | 34:2 | 2.8 | 137 ± 11 | 725 ± 243 | 1350 ± 200 | 6 ± 0.9 | 2.85 | |
| | 34:3 | 2.7 | 39 ± 21 | 74 ± 34 | 150 ± 15 | 0.67 ± 0.06 | | N.D. |
| | 34:4 | 2.59 | | 21 ± 12 | 29 ± 10 | 0.13 ± 0.05 | | N.D. |
| | 32:0 | 2.82 | | 71 ± 30 | 111 ± 34 | 0.5 ± 0.15 | | N.D. |
| | 32:1 | 2.63 | | 65 ± 30 | 88 ± 37 | 0.4 ± 0.16 | 2.97 | |
| | 32:2 | 2.51 | | 16.3 ± 3.3 | 56 ± 10 | 0.25 ± 0.04 | | N.D. |
| | 32:3 | 2.44 | | 7.2 ± 5.6 | 12.1 ± 5.4 | 0.05 ± 0.02 | | N.D. |
| | 30:0 | 2.49 | | 11.3 ± 2.6 | 14.3 ± 4.2 | 0.06 ± 0.02 | | N.D. |
| | 30:1 | 2.34 | | 22.2 ± 12.1 | 52 ± 22 | 0.2 ± 0.1 | | N.D. |
| | 30:2 | 2.17 | | 5.6 ± 2.3 | 16.9 ± 9.9 | 0.08 ± 0.04 | | N.D. |
| LPE | 24:6 | 1.79 | | | 33.2 ± 19.6 | 0.04 ± 0.02 | | N.D. |
| | 24:7 | 1.75 | | | 48 ± 3 | 0.058 ± 0.004 | | N.D. |
| | 22:4 | 1.68 | | | 111 ± 33 | 0.13 ± 0.04 | | N.D. |
| | 22:5 | 1.63 | | 637 ± 164 | 1100 ± 140 | 1.3 ± 0.17 | 1.57 | |
| | 22:6 | 1.65 | 510 ± 170 | 7900 ± 960 | 11600 ± 1200 | 14 ± 1.5 | 1.57 | |
| | 20:0 | 1.96 | | 18.8 ± 6.1 | 51 ± 16 | 0.06 ± 0.02 | 1.7 | |
| | 20:1 | 1.73 | | 81 ± 20 | 225 ± 21 | 0.27 ± 0.02 | 1.66 | |
| | 20:2 | 1.68 | | 77 ± 22 | 205 ± 42 | 0.25 ± 0.05 | 1.63 | |
| | 20:3 | 1.63 | 106 ± 26 | 776 ± 57 | 1580 ± 110 | 1.9 ± 0.1 | 1.56 | |
| | 20:4 | 1.59 | 420 ± 70 | 6400 ± 1500 | 9200 ± 600 | 11.1 ± 0.7 | 1.53 | |
| | 18:0 | 1.66 | 611 ± 51 | 9200 ± 1100 | 14300 ± 1200 | 17.3 ± 1.4 | 1.6 | |
| | 18:1 | 1.61 | | 8800 ± 800 | 14700 ± 1000 | 17.7 ± 1.3 | 1.55 | |
| | 18:2 | 1.58 | 374 ± 49 | 4300 ± 200 | 7200 ± 500 | 8.7 ± 0.6 | 1.5 | |
| | 18:3 | 1.55 | | 74 ± 25 | 153 ± 47 | 0.18 ± 0.06 | 1.5 | |
| | 16:0 | 1.57 | 1130 ± 140 | 11200 ± 360 | 21600 ± 350 | 26 ± 0.4 | 1.54 | |
| | 16:1 | 1.52 | 19.9 ± 8.2 | 340 ± 128 | 830 ± 130 | 1 ± 0.15 | 1.49 | |
| LPS | 22:5 | 1.66 | | 64 ± 28 | 144 ± 20 | 1.8 ± 0.2 | | N.D. |
| | 22:6 | 1.63 | 78 ± 20 | 564 ± 102 | 960 ± 120 | 11.7 ± 1.5 | 1.85 | |
| | 20:0 | 1.8 | | | 6.2 ± 4.3 | 0.08 ± 0.05 | | N.D. |
| | 20:1 | 1.75 | | 10.8 ± 6.1 | 24.6 ± 5.5 | 0.3 ± 0.07 | | N.D. |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 20:2 | 1.7 | | | 19.1 ± 2.9 | 0.23 ± 0.04 | | N.D. |
| | 20:3 | 1.64 | | 98 ± 9 | 154 ± 17 | 1.9 ± 0.2 | | N.D. |
| | 20:4 | 1.6 | 70 ± 22 | 424 ± 62 | 730 ± 93 | 9 ± 1.2 | | N.D. |
| | 18:0 | 1.69 | 232 ± 49 | 1780 ± 190 | 3740 ± 450 | 45.7 ± 5.6 | 1.47 | |
| | 18:1 | 1.66 | | 378 ± 43 | 695 ± 33 | 8.5 ± 0.4 | | N.D. |
| | 18:2 | 1.61 | | 160 ± 4 | 329 ± 44 | 4 ± 0.5 | | N.D. |
| | 18:3 | 1.49 | | | 12.5 ± 5.6 | 0.15 ± 0.07 | | N.D. |
| | 16:0 | 1.6 | 83 ± 30 | 634 ± 105 | 1220 ± 80 | 14.9 ± 2.9 | 1.51 | |
| | 16:1 | 1.58 | | 34.8 ± 6.9 | 84 ± 7 | 1 ± 0.1 | | N.D. |
| | 16:2 | 1.6 | | | 66 ± 12 | 0.8 ± 0.14 | | N.D. |
| LPG | 22:6 | 1.57 | 66 ± 16 | 640 ± 170 | 1670 ± 220 | 12.4 ± 1.6 | | N.D. |
| | 20:3 | 1.61 | | 46 ± 28 | 96 ± 27 | 0.72 ± 0.2 | | N.D. |
| | 20:4 | 1.57 | | 84 ± 27 | 186 ± 42 | 1.4 ± 0.3 | | N.D. |
| | 18:0 | 1.75 | | 28.6 ± 10.5 | 64.8 ± 8 | 0.48 ± 0.06 | 1.67 | |
| | 18:1 | 1.6 | 155 ± 25 | 1210 ± 110 | 2390 ± 80 | 18 ± 0.6 | 1.61 | |
| | 18:2 | 1.56 | 186 ± 50 | 2670 ± 120 | 3900 ± 640 | 29.1 ± 4.8 | 1.55 | |
| | 18:3 | 1.54 | | | 26.6 ± 15.6 | 0.2 ± 0.12 | | N.D. |
| | 16:0 | 1.79 | | 38.3 ± 14.7 | 94 ± 11 | 0.7 ± 0.08 | 1.56 | |
| | 16:1 | 1.66 | 30 ± 13.6 | 440 ± 90 | 1040 ± 170 | 7.8 ± 1.2 | | N.D. |
| | 14:0 | 1.69 | 165 ± 24 | 2560 ± 320 | 3950 ± 140 | 29.4 ± 1 | | N.D. |
| LPI | 20:3 | 1.57 | 12.8 ± 1.4 | 138 ± 70 | 232 ± 23 | 5.9 ± 0.6 | | N.D. |
| | 20:4 | 1.54 | 59.4 ± 7.2 | 857 ± 167 | 1140 ± 170 | 29 ± 4.3 | 1.76 | |
| | 18:0 | 1.62 | 165 ± 31 | 1670 ± 150 | 2120 ± 250 | 54.1 ± 6.4 | 1.83 | |
| | 18:1 | 1.56 | 23.6 ± 16 | 158 ± 51 | 280 ± 36 | 7.1 ± 0.9 | | N.D. |
| | 18:2 | 1.52 | | 18.8 ± 8.1 | 38.9 ± 4.7 | 1 ± 0.1 | | N.D. |
| | 16:0 | 1.51 | | 76 ± 30 | 114 ± 18 | 2.9 ± 0.5 | | N.D. |
| LPA | 24:6 | 1.96 | | 194 ± 30 | 553 ± 86 | 0.23 ± 0.04 | | N.D. |
| | 24:7 | 1.87 | | 117.4 ± 18.3 | 225 ± 28 | 0.09 ± 0.01 | | N.D. |
| | 22:0 | 2.06 | | 9.4 ± 2.5 | 34.3 ± 6.5 | 0.014 ± 0.003 | | N.D. |
| | 22:1 | 1.98 | | 29.8 ± 5.3 | 56 ± 16 | 0.024 ± 0.007 | | N.D. |
| | 22:2 | 1.91 | | 6.1 ± 2.2 | 12.3 ± 3.8 | 0.005 ± 0.002 | | N.D. |
| | 22:3 | 1.79 | | 11.9 ± 2.9 | 15.3 ± 2.1 | 0.006 ± 0.001 | | N.D. |
| | 22:4 | 1.75 | 25.1 ± 16.9 | 113 ± 32 | 252 ± 35 | 0.11 ± 0.01 | | N.D. |
| | 22:5 | 1.73 | 89 ± 15 | 634 ± 56 | 1090 ± 75 | 0.46 ± 0.03 | | N.D. |
| | 22:6 | 1.67 | 505 ± 127 | 3300 ± 360 | 4940 ± 520 | 2.1 ± 0.2 | 1.54 | |
| | 20:0 | 1.9 | | 107 ± 37 | 160 ± 22 | 0.07 ± 0.01 | | N.D. |
| | 20:1 | 1.83 | 43 ± 11 | 372 ± 36 | 640 ± 73 | 0.27 ± 0.03 | | N.D. |
| | 20:2 | 1.75 | 57 ± 9 | 510 ± 60 | 852.9 ± 49 | 0.36 ± 0.02 | | N.D. |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 20:3 | 1.69 | 223 ± 15 | 2050 ± 310 | 5200 ± 500 | 2.2 ± 0.2 | 1.58 | |
| | 20:4 | 1.66 | 589 ± 25 | 4690 ± 520 | 8180 ± 980 | 3.4 ± 0.4 | 1.52 | |
| | 18:0 | 1.76 | 3250 ± 520 | 25800 ± 1500 | 48000 ± 2000 | 20.3 ± 0.9 | 1.6 | |
| | 18:1 | 1.69 | 2150 ± 270 | 19500 ± 2400 | 44600 ± 1800 | 18.8 ± 0.8 | 1.54 | |
| | 18:2 | 1.63 | 2070 ± 530 | 19300 ± 3400 | 44400 ± 7100 | 18.7 ± 3 | 1.49 | |
| | 18:3 | 1.6 | 22.8 ± 5.8 | 236 ± 42 | 449 ± 97 | 0.19 ± 0.04 | 1.49 | |
| | 16:0 | 1.64 | 3760 ± 750 | 35500 ± 7000 | 73200 ± 11200 | 31 ± 4.7 | 1.52 | |
| | 16:1 | 1.59 | 190 ± 90 | 2020 ± 430 | 4000 ± 410 | 1.7 ± 0.2 | 1.46 | |
| | 16:2 | 1.45 | | 48 ± 12 | 64 ± 12 | 0.027 ± 0.005 | | N.D. |
| | 14:0 | 1.43 | 41 ± 12 | 178 ± 51 | 331 ± 61 | 0.14 ± 0.03 | | N.D. |
| SM | 28:0 | 4.07 | | 8.4 ± 3.2 | 31 ± 23 | 0.0035 ± 0.0026 | | N.D. |
| | 28:1 | 3.91 | | 19 ± 10 | 18 ± 8 | 0.0021 ± 0.0009 | | N.D. |
| | 28:2 | 3.6 | | 8.4 ± 8.2 | 17.3 ± 1.4 | 0.002 ± 0.0002 | | N.D. |
| | 26:0 | 3.61 | 6.2 ± 0.8 | 24.6 ± 2.2 | 47.3 ± 16.5 | 0.0054 ± 0.0019 | 2.96 | |
| | 26:1 | 3.39 | 4.9 ± 3.3 | 30 ± 13 | 100 ± 10 | 0.011 ± 0.001 | 2.84 | |
| | 26:2 | 3.19 | 15.6 ± 5.8 | 83 ± 4 | 128 ± 33 | 0.015 ± 0.004 | 2.67 | |
| | 26:3 | 3.02 | 70 ± 28 | 477 ± 33 | 1130 ± 280 | 0.13 ± 0.03 | 2.6 | |
| | 26:4 | 2.9 | 820 ± 57 | 4120 ± 1340 | 7900 ± 1000 | 0.89 ± 0.11 | 2.5 | |
| | 26:5 | 2.85 | 3560 ± 180 | 17700 ± 3700 | 32700 ± 5600 | 3.7 ± 0.6 | 2.37 | |
| | 26:6 | 2.68 | 3500 ± 550 | 17500 ± 3600 | 27300 ± 340 | 3.1 ± 0.04 | 2.28 | |
| | 24:0 | 3.2 | 240 ± 60 | 2240 ± 140 | 5100 ± 1000 | 0.57 ± 0.12 | | N.D. |
| | 24:1 | 2.97 | 875 ± 23 | 5230 ± 1550 | 10000 ± 1000 | 1.2 ± 0.1 | 2.55 | |
| | 24:2 | 2.9 | 2050 ± 140 | 11700 ± 3300 | 22200 ± 3900 | 2.5 ± 0.4 | 2.46 | |
| | 24:3 | 2.79 | 7590 ± 580 | 34700 ± 9400 | 72000 ± 13000 | 8.2 ± 1.4 | 2.34 | |
| | 24:4 | 2.64 | 7330 ± 840 | 39800 ± 9400 | 66000 ± 8600 | 7.5 ± 1 | 2.27 | |
| | 24:5 | 2.57 | 14300 ± 1000 | 64600 ± 17400 | 123000 ± 18000 | 14 ± 2 | 2.16 | |
| | 24:6 | 2.45 | 462 ± 57 | 1940 ± 590 | 3220 ± 330 | 0.37 ± 0.04 | 2.1 | |
| | 22:0 | 2.88 | 2340 ± 180 | 14100 ± 3500 | 24000 ± 2400 | 2.8 ± 0.3 | 2.45 | |
| | 22:1 | 2.77 | 8450 ± 230 | 51700 ± 11900 | 101000 ± 4000 | 11.5 ± 0.4 | 2.32 | |
| | 22:2 | 2.6 | 8240 ± 630 | 39500 ± 11400 | 71200 ± 9900 | 8.1 ± 1.1 | 2.26 | |
| | 22:3 | 2.51 | 6820 ± 880 | 36800 ± 9900 | 66300 ± 7600 | 7.5 ± 0.9 | 2.2 | |
| | 22:4 | 2.45 | 1710 ± 260 | 11000 ± 3600 | 17800 ± 1800 | 2 ± 0.2 | 2.09 | |
| | 22:5 | 2.31 | 76 ± 8 | 309 ± 132 | 527 ± 123 | 0.06 ± 0.01 | | N.D. |
| | 22:6 | 2.04 | 3.7 ± 1 | 30 ± 14.1 | 44 ± 20 | 0.005 ± 0.002 | | N.D. |
| | 20:0 | 2.62 | 9000 ± 1000 | 47000 ± 17000 | 83100 ± 13300 | 9.4 ± 1.5 | 2.23 | |
| | 20:1 | 2.5 | 13200 ± 1300 | 83000 ± 29000 | 128000 ± 13000 | 14.6 ± 1.5 | 2.16 | |
| | 20:2 | 2.37 | 971 ± 68 | 4700 ± 1200 | 6900 ± 800 | 0.79 ± 0.09 | 2.1 | |
| | 20:3 | 2.3 | 32 ± 12.3 | 140 ± 40 | 190 ± 19 | 0.022 ± 0.002 | | N.D. |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 18:0 | 2.38 | 640 ± 140 | 2950 ± 970 | 5780 ± 890 | 0.7 ± 0.1 | 2.22 | |
| | 18:1 | 2.3 | 128 ± 21 | 443 ± 141 | 583 ± 68 | 0.066 ± 0.008 | 2.11 | |
| | 18:2 | 2.12 | | 13 ± 4.7 | 23.2 ± 13.5 | 0.0026 ± 0.0015 | 2.03 | |
| | 16:0 | 2.15 | 290 ± 27 | 1170 ± 90 | 1520 ± 370 | 0.17 ± 0.04 | 2.1 | |
| | 16:1 | 2.08 | 5 ± 2 | 34.3 ± 10.7 | 38.3 ± 16 | 0.004 ± 0.002 | 2 | |
| | 14:0 | 1.91 | | 13.5 ± 8.4 | 16.4 ± 15 | 0.0019 ± 0.0017 | 1.93 | |
| Cer1P | 24:1 | 3.83 | | 39.4 ± 18 | 148 ± 26 | 2.3 ± 0.4 | | N.D. |
| | 24:3 | 3.43 | | 10.3 ± 3.9 | 23.5 ± 4.9 | 0.4 ± 0.08 | | N.D. |
| | 24:5 | 3.1 | | 34.7 ± 9.6 | 74.5 ± 22.3 | 1.1 ± 0.3 | | N.D. |
| | 24:6 | 2.93 | | 7.8 ± 4 | 22.5 ± 7.6 | 0.3 ± 0.1 | | N.D. |
| | 22:0 | 3.49 | | 11.3 ± 5.9 | 29.8 ± 3.3 | 0.46 ± 0.06 | | N.D. |
| | 22:2 | 3.26 | 94.4 ± 18.2 | 435 ± 76 | 989 ± 79 | 15 ± 1 | | N.D. |
| | 22:3 | 3.05 | 330 ± 43 | 1920 ± 250 | 3620 ± 390 | 55.8 ± 6 | | N.D. |
| | 22:4 | 2.87 | | 51.1 ± 10.4 | 114 ± 17 | 1.8 ± 0.3 | 3.62 | |
| | 22:5 | 2.76 | | 8.9 ± 3.3 | 20.4 ± 8.8 | 0.3 ± 0.1 | 3.31 | |
| | 22:6 | 2.72 | | 9.4 ± 2.6 | 21.6 ± 9.7 | 0.3 ± 0.1 | 3.12 | |
| | 20:1 | 3 | | 12.5 ± 3.4 | 28.5 ± 12.7 | 0.4 ± 0.2 | | N.D. |
| | 20:2 | 2.93 | 94 ± 22 | 473 ± 62 | 1080 ± 120 | 16.6 ± 1.8 | | N.D. |
| | 20:3 | 2.75 | | 23.2 ± 5.9 | 57.2 ± 11.3 | 0.9 ± 0.2 | | N.D. |
| | 20:4 | 2.56 | | | 10.4 ± 1.2 | 0.16 ± 0.03 | 3.17 | |
| | 18:0 | 2.83 | | | 8.5 ± 3.2 | 0.13 ± 0.05 | 3.78 | |
| | 18:1 | 2.67 | | 8.4 ± 3.2 | 21.3 ± 5.6 | 0.33 ± 0.09 | 3.52 | |
| | 18:2 | 2.62 | | 37 ± 12 | 95 ± 14 | 1.5 ± 0.2 | | N.D. |
| | 16:0 | 2.25 | | 43 ± 16 | 126 ± 23 | 1.9 ± 0.4 | 3.34 | |
| So1P | 18:1 | 1.44 | 274 ± 96 | 1190 ± 410 | 2470 ± 380 | 100 | | N.D. |
| Sa1P | 18:0 | 1.47 | 47.9 ± 4.1 | 312 ± 34 | 500 ± 67 | 100 | | N.D. |

[a] Dilution ratio of lipid extracts

[b] The values of peak area, normalized by IS, are mean ± SD, (n=3)

[c] The values are mean ± SD, (n=3)

**Table 3.8** Molecular species of PC, PE, PG, PI, and LPC analyzed in mouse liver by SFC/MS/MS with methylation and the comparison with nonmethylation.

| Polar lipid | Cn_Un | Methylation | | | | | Nonmethylation | |
|---|---|---|---|---|---|---|---|---|
| | | RT (min) | Peak area (1:10) | Peak area (1:2) | Peak area (1:1) | Relative area (%) | RT (min) | Detection |
| PC | 42:2 | | | | | | 3.39 | A.D.[a] |
| | 42:3 | | | | | | 3.12 | A.D. |
| | 42:4 | 3.45 | 2 ± 1.2 | 14.7 ± 9.2 | 27.4 ± 7.1 | 0.0019 ± 0.0005 | 2.98 | |
| | 42:5 | 3.27 | 10 ± 4 | 65.3 ± 19.1 | 107.4 ± 14.6 | 0.0074 ± 0.001 | 2.86 | |
| | 42:6 | 3.14 | 44.4 ± 19.5 | 89.4 ± 38.1 | 256 ± 84.9 | 0.018 ± 0.006 | 2.74 | |
| | 42:7 | 3.02 | 72.6 ± 31.9 | 341 ± 102 | 784.7 ± 126.2 | 0.054 ± 0.009 | 2.62 | |
| | 42:8 | 2.88 | 65.8 ± 26.3 | 206 ± 49.5 | 462.4 ± 26.2 | 0.032 ± 0.002 | 2.5 | |
| | 42:9 | 2.72 | 59.8 ± 30.9 | 230 ± 73.2 | 313.5 ± 162.2 | 0.022 ± 0.011 | 2.36 | |
| | 42:10 | 2.63 | 62.5 ± 34.3 | 311 ± 158 | 761.2 ± 276.5 | 0.052 ± 0.019 | 2.3 | |
| | 40:1 | 3.74 | 3.4 ± 1.5 | 24.1 ± 20.2 | 44.7 ± 24.1 | 0.003 ± 0.0017 | 3.14 | |
| | 40:2 | 3.42 | 14.8 ± 8.2 | 37 ± 23.3 | 187.7 ± 56 | 0.013 ± 0.004 | 2.99 | |
| | 40:3 | 3.27 | 48.6 ± 9 | 102 ± 33.5 | 244 ± 148.5 | 0.017 ± 0.01 | 2.72 | |
| | 40:4 | 3.07 | 217 ± 61 | 700 ± 220 | 2100 ± 490 | 0.14 ± 0.03 | 2.64 | |
| | 40:5 | 2.94 | 1620 ± 420 | 4100 ± 180 | 13900 ± 3500 | 0.95 ± 0.24 | 2.55 | |
| | 40:6 | 2.91 | 6500 ± 1100 | 15960 ± 1090 | 52300 ± 13300 | 3.6 ± 0.9 | 2.46 | |
| | 40:7 | 2.75 | 3300 ± 1800 | 17400 ± 4300 | 41300 ± 10900 | 2.8 ± 0.7 | 2.37 | |
| | 40:8 | 2.63 | 759.5 ± 97 | 2850 ± 360 | 8600 ± 1900 | 0.6 ± 0.13 | 2.28 | |
| | 38:1 | 3.25 | 480 ± 410 | 1660 ± 980 | 6000 ± 3800 | 0.41 ± 0.26 | 2.97 | |
| | 38:2 | 3.07 | 1930 ± 520 | 6860 ± 1420 | 12300 ± 2500 | 0.84 ± 0.17 | 2.85 | |
| | 38:3 | 2.92 | 4700 ± 1100 | 15700 ± 3400 | 38000 ± 8000 | 2.6 ± 0.6 | 2.59 | |
| | 38:4 | 2.89 | 13800 ± 3600 | 41800 ± 11600 | 86000 ± 20000 | 6 ± 1.4 | 2.44 | |
| | 38:5 | 2.7 | 14200 ± 2700 | 45800 ± 9900 | 126000 ± 30000 | 8.6 ± 2.1 | 2.36 | |
| | 38:6 | 2.64 | 24000 ± 3800 | 70600 ± 13000 | 196000 ± 53000 | 13.5 ± 3.7 | 2.27 | |
| | 38:7 | 2.52 | 1010 ± 390 | 3100 ± 1300 | 8800 ± 2600 | 0.6 ± 0.2 | 2.18 | |
| | 38:8 | 2.37 | 21.2 ± 11.6 | 33.1 ± 3.2 | 93.9 ± 43.5 | 0.006 ± 0.003 | 2.09 | |
| | 36:1 | 2.9 | 4760± 1040 | 18300 ± 3400 | 41000 ± 9800 | 2.8 ± 0.7 | 2.52 | |
| | 36:2 | 2.84 | 20300 ± 6500 | 58900 ± 19900 | 115000 ± 27000 | 7.9 ± 1.8 | 2.43 | |
| | 36:3 | 2.69 | 14900± 4300 | 48600 ± 16000 | 137000 ± 38000 | 9.4 ± 2.6 | 2.34 | |
| | 36:4 | 2.59 | 14300 ± 4100 | 43000 ± 18000 | 110000 ± 9000 | 7.6 ± 0.6 | 2.25 | |
| | 36:5 | 2.5 | 3900 ± 1300 | 17800 ± 700 | 35900 ± 12000 | 2.5 ± 0.8 | 2.16 | |
| | 36:6 | 2.43 | 203 ± 54 | 689 ± 363 | 1260 ± 520 | 0.088 ± 0.036 | 2.07 | |
| | 34:1 | 2.67 | 16700 ± 610 | 81800 ± 5200 | 178000 ± 55000 | 12.3 ± 3.8 | 2.33 | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 34:2 | 2.58 | 24100 ± 9200 | 95300 ± 38000 | 178000 ± 37000 | 12.2 ± 2.6 | 2.24 | |
| | 34:3 | 2.47 | 1800 ± 870 | 6100 ± 860 | 13000 ± 4000 | 0.88 ± 0.26 | 2.15 | |
| | 34:4 | 2.41 | 69.2 ± 42.5 | 324 ± 152 | 696 ± 231 | 0.048 ± 0.016 | 2.12 | |
| | 34:5 | | | | | | 2.06 | A.D. |
| | 32:0 | 2.57 | 3200 ± 600 | 12000 ± 1500 | 34000 ± 7000 | 2.3 ± 0.5 | 2.23 | |
| | 32:1 | 2.48 | 1520 ± 640 | 5200 ± 3300 | 8400 ± 1700 | 0.58 ± 0.12 | 2.14 | |
| | 32:2 | 2.37 | 184 ± 86 | 850 ± 440 | 1450 ± 390 | 0.1 ± 0.03 | 2.08 | |
| | 32:3 | 2.28 | 9 ± 3.2 | 24.8 ± 8.8 | 41.8 ± 11.6 | 0.003 ± 0.001 | 2.05 | |
| | 30:0 | 2.31 | 144 ± 29 | 673 ± 95 | 1720 ± 440 | 0.12 ± 0.03 | 2.1 | |
| | 30:1 | 2.19 | 505 ± 84 | 1980 ± 290 | 5200 ± 1900 | 0.35 ± 0.13 | 2.04 | |
| | 30:2 | 2.11 | | 60.1 ± 24.3 | 88.8 ± 19.8 | 0.006 ± 0.001 | 1.95 | |
| PE | 40:2 | | | | | | 3.03 | A.D. |
| | 40:3 | | | | | | 2.9 | A.D. |
| | 40:4 | 3.03 | | 19.6 ± 2.4 | 58.9 ± 20.7 | 0.11 ± 0.04 | 2.71 | |
| | 40:5 | 2.85 | | 190 ± 14.1 | 715 ± 132 | 1.3 ± 0.2 | 2.62 | |
| | 40:6 | 2.79 | 129 ± 50.6 | 887 ± 261 | 3400 ± 900 | 6.4 ± 1.7 | 2.57 | |
| | 40:7 | 2.67 | 189 ± 40 | 1790 ± 460 | 4900 ± 1300 | 9.1 ± 2.4 | 2.46 | |
| | 40:8 | 2.56 | | 135 ± 39 | 428 ± 199 | 0.8 ± 0.4 | 2.36 | |
| | 38:1 | 3.29 | | | 50.9 ± 45.2 | 0.06 ± 0.08 | 2.72 | |
| | 38:2 | 2.88 | | | 61 ± 39 | 0.11 ± 0.07 | 2.56 | |
| | 38:3 | 2.83 | 44 ± 25 | 268 ± 88 | 1200 ± 590 | 2.3 ± 1.1 | 2.54 | |
| | 38:4 | 2.77 | 142 ± 15 | 960 ± 250 | 5470 ± 1590 | 10.2 ± 3 | 2.42 | |
| | 38:5 | 2.59 | 226 ± 53 | 1700 ± 240 | 9000 ± 1200 | 16.8 ± 2.2 | 2.35 | |
| | 38:6 | 2.57 | 400 ± 170 | 3300 ± 1100 | 13300 ± 3500 | 24.8 ± 6.4 | 2.26 | |
| | 38:7 | 2.43 | 41 ± 30 | 205 ± 82 | 960 ± 210 | 1.8 ± 0.4 | 2.21 | |
| | 36:0 | 2.94 | | | 68.6 ± 23.3 | 0.13 ± 0.04 | 2.64 | |
| | 36:1 | 2.74 | | 823 ± 20 | 380 ± 99 | 0.7 ± 0.18 | 2.57 | |
| | 36:2 | 2.74 | 42 ± 13 | 474 ± 196 | 2300 ± 480 | 4.2 ± 0.9 | 2.5 | |
| | 36:3 | 2.6 | 99 ± 52 | 890 ± 125 | 3400 ± 500 | 6.4 ± 0.9 | 2.39 | |
| | 36:4 | 2.5 | 161 ± 73 | 1100 ± 500 | 3800 ± 600 | 7.1 ± 1.1 | 2.3 | |
| | 36:5 | 2.42 | 65 ± 67 | 333 ± 84 | 1030 ± 260 | 1.9 ± 0.5 | 2.23 | |
| | 36:6 | 2.34 | | 29.1 ± 13.4 | 52.5 ± 28.3 | 0.1 ± 0.05 | 2.11 | |
| | 34:0 | 2.72 | | 28.5 ± 12.3 | 71.7 ± 51.9 | 0.13 ± 0.1 | 2.39 | |
| | 34:1 | 2.57 | | 213 ± 61 | 682 ± 269 | 1.3 ± 0.5 | 2.32 | |
| | 34:2 | 2.47 | 62 ± 15 | 634 ± 121 | 2080 ± 280 | 3.9 ± 0.5 | 2.3 | |
| | 34:3 | 2.33 | | 61.8 ± 0.9 | 216 ± 69 | 0.4 ± 0.13 | 2.17 | |
| | 34:4 | | | | | | 2.1 | A.D. |
| | 32:0 | | | | | | 2.24 | A.D. |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 32:1 | | | | | | 2.15 | A.D. |
| | 32:2 | | | | | | 2.08 | A.D. |
| | 30:0 | | | | | | 1.66 | A.D. |
| | 30:2 | | | | | | 1.59 | A.D. |
| PG | 36:1 | | | | | | 2.64 | A.D. |
| | 36:2 | | | | | | 2.52 | A.D. |
| | 36:3 | 2.55 | | 11.5 ± 11.1 | 38.3 ± 15.3 | 20.2 ± 8.1 | 2.41 | |
| | 36:4 | 2.4 | | 31.8 ± 34.1 | 116 ± 55 | 61 ± 29 | 2.28 | |
| | 34:1 | 2.54 | | 15.1 ± 7.7 | 20.6 ± 11.2 | 10.8 ± 5.9 | 2.42 | |
| | 34:2 | 2.39 | | 8.4 ± 1.4 | 15.2 ± 10.3 | 8 ± 5.4 | 2.31 | |
| | 34:3 | | | | | | 2.17 | A.D. |
| | 32:0 | | | | | | 2.23 | A.D. |
| PI | 40:4 | | | | | | 2.78 | A.D. |
| | 40:5 | 2.79 | | 95.8 ± 73.4 | 282 ± 117 | 2.7 ± 1.1 | 2.72 | |
| | 40:6 | 2.73 | 35.7 ± 24.7 | 182 ± 25 | 357 ± 137 | 3.5 ± 1.3 | 2.69 | |
| | 40:7 | 2.66 | 77.4 ± 41.2 | 255 ± 10.4 | 704 ± 98 | 6.8 ± 1 | 2.59 | |
| | 40:8 | 2.51 | | 39 ± 19.8 | 188 ± 37 | 1.8 ± 0.4 | | N.D.[e] |
| | 38:3 | 2.71 | 100 ± 34 | 496 ± 179 | 1920 ± 450 | 18.6 ± 4.3 | 2.68 | |
| | 38:4 | 2.63 | 307 ± 203 | 2510 ± 1080 | 5400 ± 1700 | 52.3 ± 17 | 2.63 | |
| | 38:5 | 2.56 | 60.7 ± 18.2 | 183 ± 58 | 434 ± 251 | 4.2 ± 2.4 | 2.55 | |
| | 38:6 | | | | | | 2.46 | A.D. |
| | 38:7 | 2.39 | | | 26 ± 12.7 | 0.25 ± 0.12 | 2.38 | |
| | 36:1 | 2.91 | | 211 ± 20.3 | 477 ± 352 | 4.6 ± 3.4 | 2.61 | |
| | 36:2 | 2.66 | 14.2 ± 6.2 | 48.9 ± 28.3 | 184 ± 67 | 1.8 ± 0.6 | 2.58 | |
| | 36:3 | 2.45 | 35.6 ± 12.3 | 97.9 ± 6.4 | 238 ± 219 | 2.3 ± 2.1 | 2.48 | |
| | 36:4 | 2.37 | | 64.9 ± 34.4 | 119 ± 2 | 1.15 ± 0.02 | 2.45 | |
| | 36:5 | | | | | | 2.36 | A.D. |
| | 34:1 | | | | | | 2.46 | A.D. |
| | 34:2 | | | | | | 2.38 | A.D. |
| LPC | 24:4 | | | | | | 1.78 | A.D. |
| | 24:5 | 1.84 | | 7.1 ± 6.6 | 10.9 ± 15.2 | 0.02 ± 0.02 | 1.64 | |
| | 24:6 | 1.79 | 9.5 ± 5.5 | 34.3 ± 30.3 | 40.2 ± 14.6 | 0.06 ± 0.02 | 1.53 | |
| | 24:7 | 1.65 | | 35.4 ± 20.9 | 54.1 ± 24.7 | 0.09 ± 0.04 | 1.52 | |
| | 22:1 | | | | | | 1.9 | A.D. |
| | 22:2 | | | | | | 1.81 | A.D. |
| | 22:3 | | | | | | 1.67 | A.D. |
| | 22:4 | 1.71 | 4.1 ± 2.9 | 21.4 ± 5.2 | 89 ± 25 | 0.14 ± 0.04 | 1.57 | |
| | 22:5 | 1.65 | 85.1 ± 14.1 | 495 ± 29 | 847 ± 51 | 1.33 ± 0.08 | 1.54 | |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 22:6 | 1.64 | 812 ± 78.6 | 4870 ± 430 | 7700 ± 620 | 12.1 ± 1 | 1.51 | |
| 20:0 | | | | | | 1.69 | A.D. |
| 20:1 | 1.79 | 32 ± 9.3 | 107 ± 36 | 205 ± 59 | 0.32 ± 0.09 | 1.63 | |
| 20:2 | 1.71 | 30.3 ± 6 | 154 ± 35 | 257 ± 26 | 0.41 ± 0.04 | 1.59 | |
| 20:3 | 1.65 | 168 ± 17.9 | 1000 ± 160 | 1790 ± 90 | 2.8 ± 0.1 | 1.54 | |
| 20:4 | 1.62 | 651 ± 33.3 | 3900 ± 200 | 6300 ± 400 | 9.9 ± 0.7 | 1.49 | |
| 18:0 | 1.72 | 1330 ± 250 | 5760 ± 340 | 8400 ± 600 | 13.2 ± 0.9 | 1.59 | |
| 18:1 | 1.65 | 1350 ± 65 | 6500 ± 300 | 9500 ± 610 | 15 ± 1 | 1.53 | |
| 18:2 | 1.59 | 1370 ± 180 | 6800 ± 250 | 11000 ± 300 | 17.4 ± 0.5 | 1.49 | |
| 18:3 | 1.57 | 17 ± 14 | 80.5 ± 21 | 120 ± 21 | 0.18 ± 0.03 | 1.46 | |
| 16:0 | 1.6 | 2500 ± 250 | 10500 ± 990 | 16300 ± 1200 | 25.7 ± 1.8 | 1.51 | |
| 16:1 | 1.55 | 118 ± 22 | 371 ± 84 | 790 ± 170 | 1.2 ± 0.3 | 1.48 | |
| 16:2 | | | | | | 1.43 | A.D. |
| 14:0 | 1.53 | 8.4 ± 2.9 | 24.6 ± 10 | 56 ± 29.3 | 0.09 ± 0.05 | 1.43 | |

[a] Additional detection

**Table 3.9** Molecular species of Cer, So, and Sa analyzed in mouse liver by SFC/MS/MS with nonmethylation.

| Polar lipid | Cn_Un | Nonmethylation | | | | |
|---|---|---|---|---|---|---|
| | | RT (min) | Peak area (1:10) | Peak area (1:2) | Peak area (1:1) | Relative area (%) |
| Cer | 26:0 | 4.06 | | 7.4 ± 2.3 | 17.2 ± 4.3 | 0.17 ± 0.04 |
| | 26:1 | 3.75 | | 4.6 ± 1.5 | 11.4 ± 3.2 | 0.11 ± 0.03 |
| | 26:5 | 3.4 | | 2.1 ± 1.9 | 7.3 ± 4 | 0.07 ± 0.04 |
| | 26:6 | 3.2 | | 23 ± 8 | 59.4 ± 14.3 | 0.6 ± 0.1 |
| | 24:0 | 3.56 | 192 ± 38 | 900 ± 320 | 2800 ± 900 | 28.3 ± 9.1 |
| | 24:1 | 3.33 | 940 ± 110 | 3400 ± 1800 | 5210 ± 1820 | 52.7 ± 18.4 |
| | 24:2 | 3.13 | | 290 ± 110 | 530 ± 370 | 5.3 ± 3.7 |
| | 24:3 | 2.99 | | | 8.9 ± 4.5 | 0.09 ± 0.05 |
| | 24:4 | 2.79 | | | 3.1 ± 2.5 | 0.03 ± 0.02 |
| | 24:5 | 2.7 | | 4.1 ± 2.8 | 7.2 ± 3.3 | 0.07 ± 0.03 |
| | 22:0 | 3.16 | 93 ± 25 | 420 ± 110 | 810 ± 130 | 8.2 ± 1.3 |
| | 22:1 | 2.97 | | 28.1 ± 11.3 | 48 ± 14 | 0.5 ± 0.1 |
| | 20:1 | 2.83 | | 43.7 ± 16.2 | 85 ± 19 | 0.9 ± 0.2 |
| | 20:2 | 2.7 | | | 9.2 ± 5.6 | 0.09 ± 0.06 |
| | 18:0 | 2.52 | 9 ± 5.8 | 34.2 ± 11.2 | 62 ± 9 | 0.6 ± 0.1 |
| | 16:0 | 2.28 | 32 ± 9 | 109 ± 52 | 195 ± 37 | 2 ± 0.4 |
| | 16:1 | 2.12 | | | 4.8 ± 2.2 | 0.05 ± 0.02 |
| | 14:0 | 2.11 | | 6 ± 3 | 11.2 ± 4.1 | 0.11 ± 0.04 |
| So | 18:1 | 1.72 | 2.2 ± 0.4 | 9.1 ± 3.6 | 15.8 ± 2.6 | 100 |
| Sa | 18:0 | 1.76 | 1.5 ± 0.4 | 4.5 ± 0.9 | 8.4 ± 1.5 | 100 |

### 3.4 Discussion

This study applied TMSD methylation to improve the peak shape of PS and PA for the comprehensive profiling of PLs, LPLs, and SLs. TMS silylation was insufficient to apply for PA and PS. Thus, other derivatization that is especially suitable for the phosphate group was considered. In the consideration of a suitable derivatization, methylation was selected to target the phosphate group. Phosphate group consists in six PLs, six LPLs, and several SLs so that methylation is applicable to these polar lipids. As the methylation reagent, diazomethane is a conventional reagent can be used in the neutral media at room temperature. However, this reagent is inconvenient to use because it should be prepared every time before using, and it needs great care to its dangerous properties such as explosive and carcinogenic. Comparing to the diazomethane, TMSD is relatively safe and easy to treat. It also needs no working-up procedure. In particular, its utility for phosphate was reported in the previous paper [135]. Thus, TMSD was applied for polar lipid profiling. Upon methylation, the hydrophobicity of polar lipids was increased so that SFC is effective for the analysis of methylated polar lipids. By SFC, high-throughput and simultaneous analysis of polar lipids was accomplished. MS/MS also provided the highly accurate and reliable quantification.

Since this is the first attempt to include an additional methylation step in polar lipid profiling by SFC/MS/MS, the MRM conditions were optimized for the analysis of methylated polar lipids. By the product ion scan, the precursor ion and product ion were selected. As the product ion, several fragment parts such as head group, the neutral loss of head group, and the sphingoid base were detected according to the polar lipids. In the MRM transitions, CV and CE values were optimized based on the intensity of compounds. After establishing the MRM conditions, the temperature and time were tested for TMSD methylation. Slight differences were observed under the different sets of conditions employed. Finally, TMSD methylation was optimized at 50°C for 10 min for the most efficiency in polar lipids. This condition is simple and fast so that it is effective for the practical use.

In the test of comparing several columns, Inertsil ODS-4 column (250 × 4.6 mm ID; 5 μm, GL Sciences) was selected for the profiling of methylated polar lipids. This column that has the end-capping was effective to analyze these polar lipids with sharp peak shapes. In SFC, the peak shape and separation also depend on the modifier ratio so that it was optimized at 20-25% for 5 min. Upon TMSD methylation, the peak tailing of PS, PI, PA, LPS, LPI, LPA, Cer1P, So1P, and Sa1P was suppressed. The phosphate in PA, LPA, and phospho-SLs such as Cer1P, So1P, and Sa1P has the high metal-affinity as the main factor to cause the peak tailing of these lipids. The methylation blocked successfully the hydroxyl groups in phosphate of these lipids. In the analysis of PS and LPS that have the metal-affinity, the carboxyl group in serine was also methylated by TMSD so that the peak shape of these lipids was improved. Furthermore, the peak shape of PI and LPI was advanced because their polarity was decreased by methylation. Other lipids such as PC, PE, PG, LPC, LPE, LPG, and SM showed the sharp peak shape by using both methylation and nonmethylation. Finally, methylation was proved as being effective to improve simultaneously the peak shape of various polar lipids.

In the validation study, this method showed high reproducibility and high reliability. In addition, the detection sensitivity of PS, PG, PA, LPS, LPG, LPI, LPA, Cer1P, So1P, and Sa1P improved considerably upon methylation, but those of other polar lipids changed only slightly. There was no improvement in the LODs of PI and choline- or ethanolamine-containing lipids such as PC, PE, LPC, LPE, and SM upon methylation. The low efficiency of methylation or ionization might result in these results. However, the peak shape and intensity of these polar lipids were reasonably good even in the use of methylation. Actually, in the most biological samples, PC, PE, and SM also have normally high-abundance comparing to other PLs such as PI and PG. Thus, the increased LODs of PC, PE, and SM have no problem for the practical analysis of a biological sample.

The methylation efficiency of each polar lipid in a mixture was also characterized to confirm whether methylation is useful for polar lipid profiling. According to the molecular

structure, the number of adducted methoxy groups differed (one or two). As a result, most polar lipids were effectively methylated (> 74.9%). However, PC, LPC, and SM were not methylated well, and the peak area for the nonmethylated compounds was higher than that for the methylated ones. It seems that the low efficiency of methylation is affected by the structure of choline. The choline which is capable of rotating freely to stable its structure can hinder sterically the methyltaion of polar lipids so that its efficiency might be decreased. Although the low efficiency was shown in these lipids, this method could be used for the simultaneous profiling of various polar lipids with high detection sensitivity.

The applicability of this method for the analysis of polar lipids in a biological sample was examined by using it for the analysis of mouse liver. For detail characterization of various molecular species in each polar lipid class, a number of MRM transitions were used. In the MRM data, undesirable noises were detected in addition to the signals from target compound. For this reason, chromatography was deemed necessary to separate and identify diverse lipids. By using an ODS column, various species of each polar lipid were separated according to their Cn and Un. For the compound with a higher Cn and a lower Un, the RT increased. For data analysis, each peak was selected on the basis of a reasonable RT with high repeatability. In particular, in the SM analysis, the selected fragment ion in the MRM transition is only a head group, and this analysis is insufficient to identify the specific sphingoid base and FA composition. Therefore, in the chromatographic separation, SM (d18:1-17:0) was used as the IS for reasonable identification based on the RT of species. Furthermore, the RT of each IS and standard was used to identify various species of other polar lipids.

In the high-throughput analysis, several polar lipids co-eluted in 6 min, and this can cause the ionization suppression of compounds. According to the kinds of lipid classes and their FA compositions, the responses to the ionization suppression must be different, and it can hinder the sensitive and reproducible analysis. Therefore, the normalization of peak areas based on IS was applied for the reproducible analysis. Furthermore, this method offered

high-resolution with sharp peaks and advanced the sensitivity to detect high-abundance and low-abundance species of polar lipids. In the analysis of PS by the nonmethylation method, severe peak tailing was observed for several species, and the peak shape was not sufficient for selecting the desired peaks and calculating the peak areas. However, upon methylation, various species of PS could be analyzed with improved peak shapes. In particular, sensitive and reliable quantification of low-abundance molecular species could be achieved in the case of PS, PA, LPS, LPA, Cer1P, So1P, and Sa1P. Furthermore, several minor species of LPE, LPG, LPI, and SM were additionally analyzed. Despite the low methylation efficiency and no advanced detection sensitivity of SM, seven minor species having highly unsaturated FA moieties were additionally analyzed.

### 3.5 Conclusions

In this study, SFC/MS/MS with methylation was used for the comprehensive analysis of various polar lipids. High-throughput and high-resolution analysis of polar lipids was accomplished by SFC. Highly accurate and reliable quantification was also obtained by MS/MS. The peak shape and detection sensitivity of several lipids were improved upon methylation. The chemical nature of polar lipids in SFC changed because of the adducted methoxy groups in the phosphate, and hence, peak tailing was suppressed. With this method, no prefractionation of lipids in a mixture is required. Furthermore, the comprehensive analysis of nineteen polar lipids with high reproducibility and reliability is possible; the peak shape and detection sensitivity of several polar lipids improved well, and detailed high-throughput quantification of various molecular species in each polar lipid is realized. In lipidomics, this method is expected to be useful for the quantification of polar lipids in biological samples such as plasma and tissues. Furthermore, the simultaneous profiling of PLs, LPLs, and SLs is helpful to obtain the detailed phenotype.

# Chapter 4

# General conclusions and future prospects

In this thesis, two methodologies based on SFC/MS/MS with derivatizations (silylation and methylation) were constructed for polar lipid profiling to obtain the detail phenotype of biological samples. Especially, in lipidomics, the peak tailing of several polar lipids such as acidic PLs (e.g. PS, PA, etc.) was the severe problem in the traditional analytical methods. This peak tailing must be suppressed because it causes the low detection sensitivity of these polar lipids. The broaden peak shape lowered the intensity of a target compound so that the peak tailing leads to no detection of low-abundance polar lipids in spite of the presence of these lipids in a mixture. Therefore, the peak shape must be sharpened for the comprehensive polar lipid profiling. It was reported that the metal-affinity of head groups in polar lipids is the main factor of peak tailing. Thus, two previous methods modified the mobile phase or column condition to suppress the peak tailing, but several demerits are still left. For more effective improvement in the peak shape of polar lipids having severe tailing, this study applied firstly the chemical derivatization to modify the characteristic of analyte (polar lipids) for blocking its metal-affinity. The hydrophobicity of polar lipids was increased by derivatizing the several functional groups such as hydroxyl, carboxyl, and phosphate groups. Thus, SFC was suitable to analyze the derivatized polar lipids because of its utility for the analysis of hydrophobic compounds. Furthermore, high-selective and high-sensitive analysis of various lipid species was obtained by MS/MS.

In chapter 2, an effective analytical method based on SFC/MS/MS with silylation was developed to identify and quantify ten polar lipids in a biological sample. In the investigation of several silylating reagents, TMSI was selected for silylation. Analysis conditions such as column, modifier ratio, and MRM transitions were optimized to analyze the silylated polar lipids. Validation study proved the high reproducibility, high reliability, and high sensitivity of this method. Upon silylation, the peak shape and detection sensitivity of several polar lipids

improved. Hydroxyl groups were mainly silylated by TMSI, and as a result, the analysis of PI was advanced dramatically. In the analysis of a biological sample, the sheep plasma was used for polar lipid profiling to prove the utility of this method. As a result, 79 molecular species of polar lipids (16 PIs, 20 LPCs, 5 LPEs, 5 LPAs, 1 LPI, 31 SMs, and So1P) were analyzed successfully. Comparing to nonsilylation, 27 lipid species (10 PIs, 2 LPCs, 2 LPEs, 5 LPAs, 1 LPI, 6 SMs, and So1P) were additionally analyzed. These results indicated that the silylation is useful to analyze the low-abundance polar lipid species which are difficult to detect by nonsilylation methods. The advanced polar lipid profiling is effective to characterize the polar lipid metabolism and to study the functions of polar lipids in biological samples. However, silylation was insufficient to derivatize the phosphate which is one of main factors to cause peak tailing. The analysis of PS as well as PA was not advanced by the silylation using TMSI. Therefore, more effective derivatization is required to block the phosphate group to obtain the comprehensive polar lipid profiling.

In chapter 3, TMSD methylation was applied to develop an effective analytical method by SFC/MS/MS for polar lipids with sharp peak shape and advanced sensitivity. Methylation is an effective method to derivatize the phosphate. Of several reagents, TMSD was simple and useful to methylate the phosphate in six PLs, six LPLs, and four SLs. The chromatographic conditions (column and modifier) and methylation conditions (time and temperature) were optimized to analyze the methylated polar lipids. This method showed the high reproducibility, high reliability, and high sensitivity. The peak shape and detection sensitivity of several polar lipids including PS and LPS were improved by methylation. Next, in the analysis of mouse liver, 287 polar lipid species (40 PCs, 23 PEs, 16 PSs, 4 PGs, 12 PIs, 33 PAs, 17 LPCs, 16 LPEs, 14 LPSs, 10 LPGs, 6 LPIs, 22 LPAs, 34 SMs, 18 Cers, 18 Cer1Ps, So, So1P, Sa, and Sa1P) were analyzed. Besides, more 84 species (4 PSs, 24 PAs, 3 LPEs, 11 LPSs, 6 LPGs, 4 LPIs, 13 LPAs, 7 SMs, 11 Cer1Ps, So1P, and Sa1P) were analyzed than nonmethylation. It demonstrated that TMSD methylation is useful for the comprehensive polar lipid profiling. In

the comparison of LODs, the methylation provided the more advanced detection sensitivity of polar lipids than silylation. Furthermore, the analysis of PS and PA was advanced successfully by methylation. This indicated that methylation is better than silylation for the comprehensive polar lipid profiling. In particular, in the analysis of PI, silylation was better to improve the sensitivity than methylation. Finally, silylation and methylation are proved to be useful for the detail profiling of polar lipids according to the target lipids and purpose.

The data of lipidomics analysis has relied on the performance of used analytical system. For example, the range of applicable compounds is limited to the developed system. The number of detected compounds also changes according to the sensitivity of system. Thus, the sensitive and simultaneous analytical system is critical for the comprehensive determination of polar lipids. Simultaneous analysis is useful to find the alterations of flux in diverse polar lipids in parallel. In the separation of polar lipid species by chromatography, if the number of target species is increased, the required analysis time is also increased for the high-resolution. The analysis time is relatively long to analyze PLs by LC/MS, and the time-consuming can hinder the practical analysis of biological samples when the samples are too many. Shotgun lipidomics analysis based on only MS/MS has also been developed for the high-throughput and sensitive quantification of lipids species. However, no separation by chromatography can cause the ionization suppression, and the sensitivity of several lipid species can be decreased. Therefore, it is challenging to develop the simultaneous, high-throughput, and high-resolution system for polar lipid profiling. In this study, SFC was used successfully to achieve this goal. High-selective analysis using MRM also provided the detail and sensitive quantification of not only high-abundance but also low-abundance species of polar lipids. Especially, the useful derivatizations (silylation and methylation) were characterized detail for the analysis of acidic PLs and phospho-SLs. The sharp peak shape of these lipids provided the improved detection sensitivity. In the analysis of a lipid mixture in biological samples, the improved detection sensitivity is critical to detect the low-abundance compounds in spite of the hindrance of

noises. The resolution of each peak is also advanced by sharp peak shape, and various polar lipid species having different Cn and Un can be separated effectively. Finally, SFC/MS/MS with derivatization is expected to be useful to achieve the detail and comprehensive profiling of polar lipids in biological samples for lipidomics.

Each lipid has its own roles in a certain region of living organisms. The altered lipid causes the different execution of its functions and it is related to various enzymes that role for the biosynthesis and hydrolysis of lipids. Polar lipids have high diversity according to the head groups and FA composition, and the analysis of these lipids was studied widely in recent decades. Different head groups in polar lipids have specific chemical natures and roles. Their localization in organisms such as cell and tissues is also specific to the kinds of polar lipids. Furthermore, the composition of FAs having different Cn and Un have effects on the rigidity of cell membrane and the affinity to enzymes. Based on the detail determination of various polar lipids, it is possible to find the flux of lipids and specificity of interaction with related enzymes. Therefore, the qualitative and quantitative analysis of various polar lipids is helpful to understand the lipid metabolism. In the quantitative analysis of various lipids, the relative quantification has been applied widely. According to each lipid class, the relative area (peak area of a molecular species/total peak area of all the molecular species) has been widely used to characterize the quantification of each lipid. On the other hand, the absolute quantification of various lipid species in biological samples is practically difficult. For this, it is required to use the stable isotope ($^{13}C$) labeling and lipid extraction in a solution spiked with unlabeled standards [136]. Especially, in the non-target analysis, it is difficult to select a certain standard compound and to obtain the stable collection of biological samples. Due to these limitations, not absolute quantification but relative quantification is normally performed to phenotype the biological samples.

Recently, the interests in metabolic changes of lipids in human diseases have been increased. Comprehensive polar lipid profiling is useful to characterize the aberrant lipid

metabolism in human diseases. Several biological samples such as cells (e.g. blood, etc.) and tissues (e.g. liver, brain, etc.) were used widely for the study of biomarkers and drug development. In lipidomics, the lipid profiling is limited to the performance of analytical system so that it is required to develop a more effective method. Especially, previous methods for polar lipid profiling have the peak tailing or the long analysis time (90 min). Finally, in this study, an effective analytical system for the high-throughput and simultaneous analysis of PLs, LPL, and SLs was developed by SFC/MS/MS with derivatization. This is the first report on the simultaneous profiling of nineteen polar lipids (six PLs, six LPLs, and seven SLs) with sharp peak shape only in 6 min. This method should be useful for the high-throughput and comprehensive phenotyping of biological samples.

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# Appendix

## Dual derivatization (methylation and silylation)

In this study, TMS silylation and TMSD methylation were characterized respectively to improve the polar lipid profiling. In the derivatization of polar lipids, the hydroxyl group was silylated by TMSI and the carboxyl and phosphate groups were methylated by TMSD. As a result, TMSD methylation was sufficient to obtain the comprehensive profiling of six PLs, six LPLs, and seven SLs. However, the methylation couldn't advance the PI analysis successfully because it cannot derivatize five hydroxyl groups of the inositol. TMS silylation was suitable to improve the PI analysis. Of course, these two derivatizaitons (methylation and silylation) are applicable for polar lipid profiling according to the target compounds. However, in order to optimize the most effective derivatization for polar lipid profiling, the dual derivatization combining methylation and silylation should be confirmed.

These two derivatizations were very simple and easy to use. The optimized condition of TMSD methylation was at 50°C for 10 min, and those of TMS silylation was at 37°C for 30 min. After the MeOH extraction, the lipid extracts was applied directly to TMSD methylation. The methylated sample was desolvated by $N_2$ gas, and then it was silylated by TMSI. This dual derivatization was simple and fast to use even though two methods were combined for it. In particular, this method was applied to the PI analysis. The modification of PI (12:0-13:0) structures by methylation and silylation was described in Figure 1. By TMSD, a methyl group was attached to the phosphate group. After methylation, five TMS groups were attached to hydroxyl groups of inositol by TMSI.

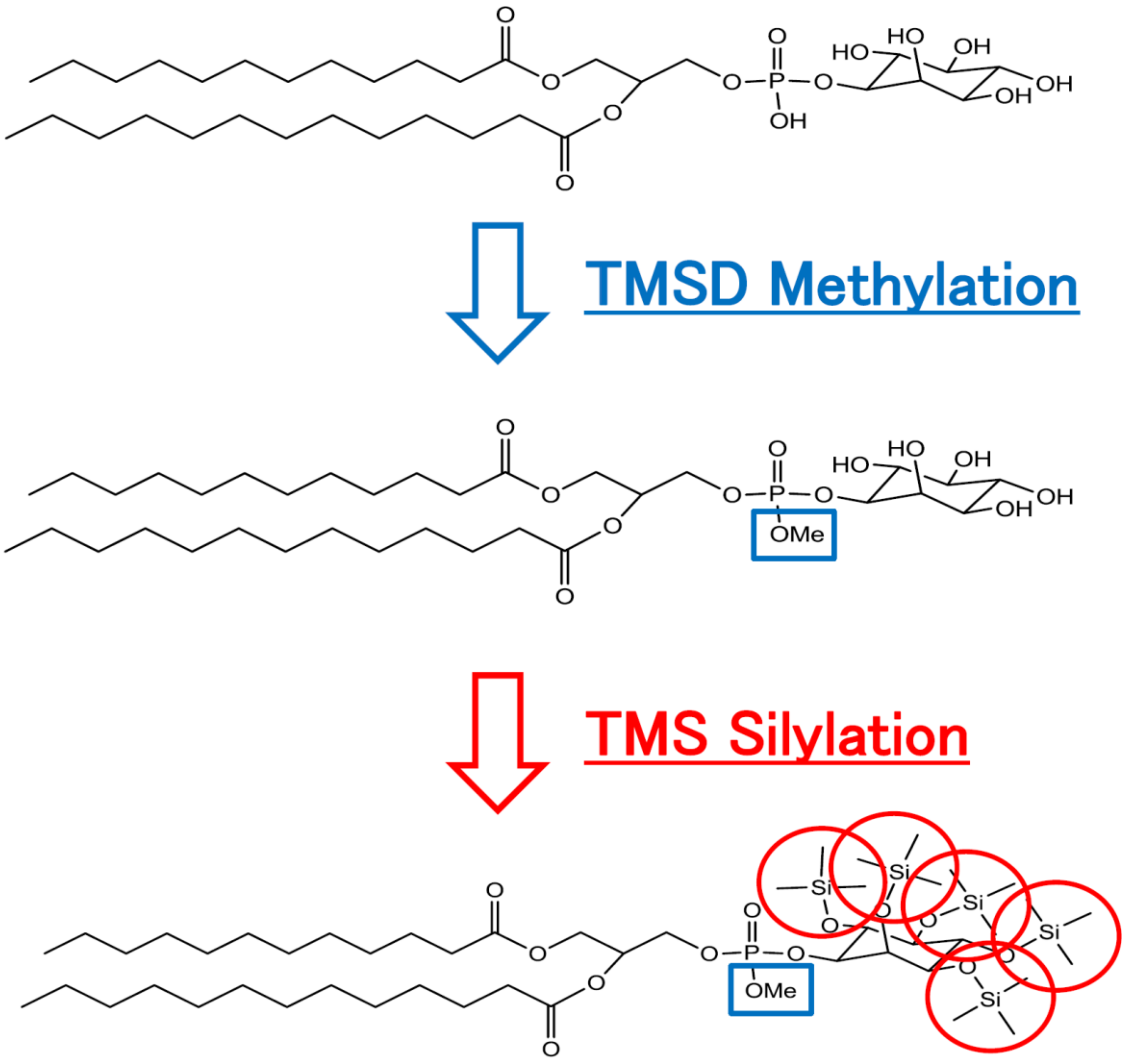


**Figure 1** Modified structure of PI (12:0-13:0) by TMSD methylation and TMS silylation

For the analysis of dual derivatized PI, MRM transition was constructed by selecting *m/z* values of precursor ion and product ion. Furthermore, when the values of CV and CE are 30 and 25 respectively, the dual derivatized PI was analyzed successfully in MRM. In order to find the utility of this derivatization, it should be compared with methylation and silylation. In the totally same analytical conditions using Inertsil ODS-4 column (250 × 4.6 mm ID; 5 µm, GL Sciences) in the modifier ratio (20-25% for 5 min), three samples including methylated, silylated, and dual derivatized PI (12:0-13:0) were analyzed (Figure 2).

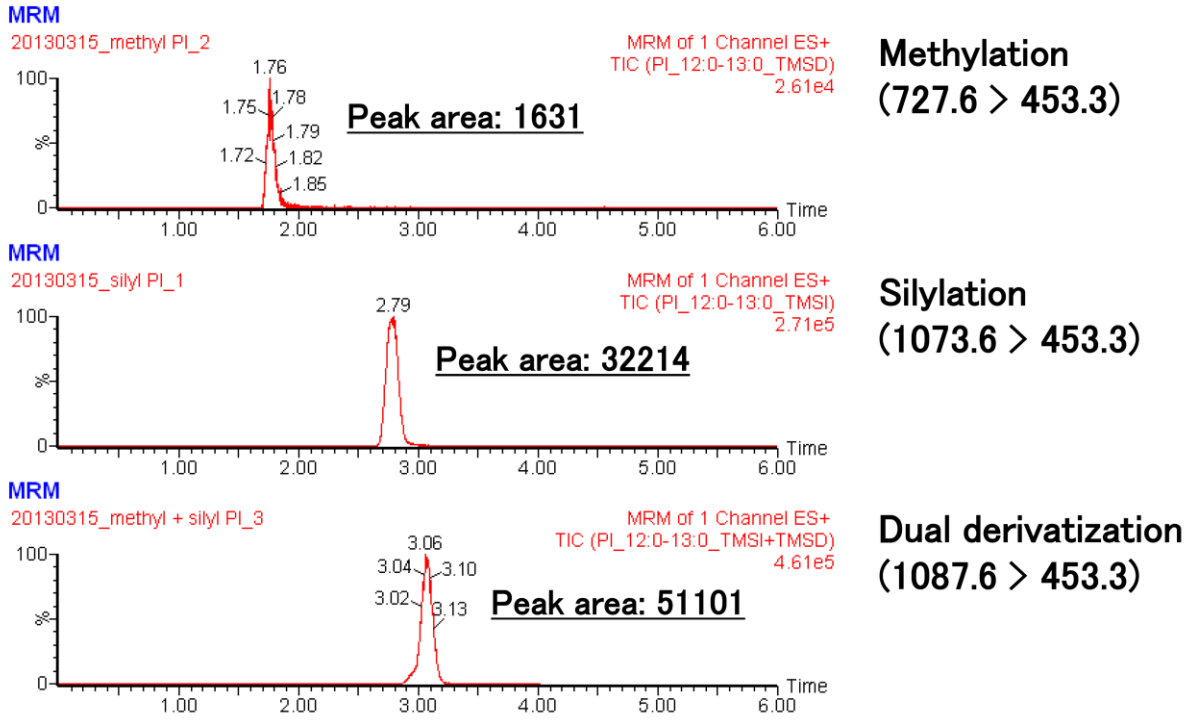


**Figure 2** MRM data of methylated, silylated, and dual derivatized PI (12:0-13:0)

In each MRM, three derivatized PIs were analyzed with sharp peak shape. According to the kinds of used derivatization, the degree of modified hydrophobicity was different so that their RTs were also different based on the increased hydrophobicity (methylated PI, 1.76 min; silylated PI, 2.79 min; and dual derivatized PI, 3.06 min). By applying three different derivatizations to the same amount of PI standard, the peak area of derivatized compounds was also calculated (methylated PI, 1631; silylated PI, 32214; and dual derivatized PI, 51101). As a result, the dual derivatization provided the highest sensitivity of a compound. The utility of this method depends on the structure of each polar lipid. Finally, the dual derivatization will be effective for the analysis of not only PI but also other polar lipids.

## Published papers

### Original papers for PhD thesis

1. Lee J. W., Yamamoto T., Uchikata T., Matsubara A., Fukusaki E., Bamba T., Development of a polar lipid profiling method by supercritical fluid chromatography/mass spectrometry, *Journal of Separation Science*, **34**, 3553-3560 (2011).
2. Lee J. W., Nishiumi S., Yoshida M., Fukusaki E., Bamba T., Simultaneous profiling of polar lipids by supercritical fluid chromatography/tandem mass spectrometry with methylation, *Journal of Chromatography A*, **1279**, 98-107 (2013).

### Additional publications

1. Lee J. W., T., Uchikata T., Matsubara A., Nakamura T., Fukusaki E., Bamba T., Application of supercritical fluid chromatography/mass spectrometry to lipid profiling of soybean, *Journal of Bioscience and Bioengineering*, **113**, 262-268 (2012).
2. Lee J. W., T., Fukusaki E., Bamba T., Application of supercritical fluid carbon dioxide to the extraction and analysis of lipids, *Bioanalysis*, **4**, 2413-2422 (2012).
3. Bamba T., Lee J. W., Matsubara A., Fukusaki E., Metabolic profiling of lipids by supercritical fluid chromatography/mass spectrometry, *Journal of Chromatography A*, **1250**, 212-219 (2012).

## Academic conference

1. ○Jae Won Lee, Takato Uchikata, Atuki Matubara, Takuji Nakamura, Eiichiro Fukusaki, Takeshi Bamba, "Application of supercritical fluid chromatography/mass spectrometry (SFC/MS) to lipid profiling of soybean", 61th SBJ Annual Meeting, Nagoya, Japan, Sep. 2009.
2. ○Takeshi Bamba, Jae Won Lee, Takato Uchikata, Atuki Matubara, Takuji Nakamura, Eiichiro Fukusaki, "Application of supercritical fluid chromatography/mass spectrometry (SFC/MS) to lipid profiling of soybean", 58th ASMS Conference on Mass Spectrometry, May 2010.
3. ○Takeshi Bamba, Jae Won Lee, Takato Uchikata, Atuki Matubara, Takuji Nakamura, Eiichiro Fukusaki, "Metabolic profiling of soybean lipids by supercritical fluid chromatography/mass spectrometry", SFC 2010, Stockholm, Sweden, Sep. 2010.
4. ○Takeshi Bamba, Jae Won Lee, Takashi Yamamoto, Takato Uchikata, Atsuki Matsubara, Eiichiro Fukusaki, "Development of polar lipid profiling method by supercritical fluid chromatography/mass spectrometry", 59th ASMS Conference on Mass Spectrometry, June 2011.
5. ○Takeshi Bamba, Jae Won Lee, Takashi Yamamoto, Takato Uchikata, Atsuki Matsubara, Eiichiro Fukusaki, "Development of polar lipid profiling method by supercritical fluid chromatography/mass spectrometry (SFC/MS)", SFC 2011, New York, USA, July 2011.
6. ○Jae Won Lee, Takashi Yamamoto, Takato Uchikata, Atsuki Matsubara, Eiichiro Fukusaki, Takeshi Bamba, "Development of polar lipid profiling method by supercritical fluid chromatography/mass spectrometry", 59th Annual conference on mass spectrometry, Osaka, Japan, Sep. 2011.
7. ○Jae Won Lee, Takashi Yamamoto, Takato Uchikata, Atsuki Matsubara, Eiichiro Fukusaki, Takeshi Bamba, "Development of polar lipid profiling method by supercritical fluid chromatography/mass spectrometry (SFC/MS)", 6th Metabolome symposium, Osaka,

Japan, Oct. 2011.

8. ○Jae Won Lee, Shin Nishiumi, Masaru Yoshida, Eiichiro Fukusaki, Takeshi Bamba, “Simultaneous profiling of polar lipids by supercritical fluid chromatography/tandem mass spectrometry with methylation”, 60th ASMS Conference on Mass Spectrometry, Vancouver, Canada, May 2012.
9. ○Jae Won Lee, Shin Nishiumi, Masaru Yoshida, Eiichiro Fukusaki, Takeshi Bamba, “Simultaneous profiling of polar lipids by supercritical fluid chromatography/tandem mass spectrometry with methylation”, 19th International Mass Spectrometry Conference, Kyoto, Japan, Sep. 2012.

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