TLC-based MS Imaging Analysis of Glycosphingolipids and Glycerin Fatty Acid Esters after 1,2-Dichloroethane Washing

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Reasons for Urgent Publication

For “Rapid Communications” present the reason(s) for urgent publication here.

In this study, we applied 1,2-dichloroethane extraction for the removal of backgrounds from separated lipids on thin-layer chromatography plates or followed by blotted hydrophilic polyvinylidene fluoride (PVDF) membranes for mass spectrometry imaging.

The established method is simple, rapid, and easily reproducible. Therefore, we urge the publication of this report as soon as possible.
Abstract

Matrix-assisted laser desorption/ionization-based mass spectrometry imaging (MSI) of separated lipids on thin-layer chromatography (TLC) plates or followed by blotted hydrophilic polyvinylidene fluoride (PVDF) membranes has become a powerful tool in lipidomic analyses. However, background peaks in MS spectra often cover lipid peaks in a low amount/ionization effect; consequently, only low intensities/resolutions MSI are obtained. To address the aforementioned problem, we attempted 1,2-dichloroethane pre-washing of TLC plates before development and found that backgrounds could successfully be removed from the TLC plate or PVDF membrane.
Introduction

Lipid composition in the cell membrane constantly changes in the environment, such as stress, development, aging, and diseases\textsuperscript{1-5}. The fatty acid chain length, number and position of double bonds and hydroxy residues, and head groups, including oligosaccharide moieties are involved in the regulation of hydrophobicity, conformational changes, or interactions between lipid–lipid or lipid–other compounds\textsuperscript{6-9}. Therefore, membrane fluidity, plasticity, thickness, receptor distribution, and permeability of low molecular weight compounds are easily regulated by accumulation/dissociation and forming of lipid rafts (membrane microdomains) and rapid changings in lipid expression, as described\textsuperscript{10-12}. Although analysis of the detailed structural characterization and lipid composition are required for elucidating biological functions, it is still difficult to use traditional extraction, purification, and analytical methods\textsuperscript{13,14}.

Normally, several column chromatography techniques are used for isolation, and thin-layer chromatography (TLC), gas chromatography, nuclear magnetic resonance, and mass spectrometry (MS) are used for characterization\textsuperscript{15}. MS has recently become one of the most powerful tools for lipid analysis, because the structural information of lipids can be rapidly and easily obtained with high sensitivity and throughput\textsuperscript{16}. Matrix–assisted laser desorption/ionization (MALDI) is the common, rapid, and convenient method for MS even containing high amounts of salts in the biological sources compared to other ionization methods\textsuperscript{17,18}. MALDI time-of-flight (TOF) MS imaging (MSI) techniques have been developed for the visualization of biological molecules, particularly lipids, in organelle sections\textsuperscript{19,20}. In the common procedure, the sections on the indium–tin–oxide coated glass plate were sprayed with matrix solution and thereafter analyzed by MALDI-TOF MS spectrometry. The precise distribution or
localization of lipids on the section can be easily obtained by MS image reconstruction without any other extraction or purification steps. However, regionally specific ion suppression in MALDI MSI has often been discussed. To address this problem, lipids need to be extracted and analyzed after separation. The MALDI–TOF MSI technique has also been applied to separated lipids on TLC plate, referred to as TLC–MSI. The retention value (Rf value) on TLC and the \( m/z \) value in the MS spectra could be compared by TLC-MSI; therefore, the assignments of each lipid could easily be completed. Although the aforementioned method is simple, it requires the separation of crude lipids extracted from cells or organs (its sections) on TLC and thereafter performing MSI analysis. Blotting from TLC plates to polyvinylidene difluoride (PVDF) membranes often yields better MSI results depending on the procedure of sample preparation or ionization efficiency of lipids. This method is known as TLC–blot MSI. TLC– or TLC–blot MSI are powerful tools for lipid analysis; however, there are still unaddressed problems, and the peak intensities drastically change depending on the background derived from the TLC plate. We previously developed a method to remove the background from lipid samples using 1,2–dichloroethane (DCE). In the present study, we investigated the optimum conditions for improving background suppression in TLC (–blot) MSI of various types of lipids using DCE washing.

Experimental

Samples

Glucosylceramide (GlcCer) prepared from bovine milk was purchased from Nagara Science (Japan), and 2,5-dihydroxy benzoic acid (DHB) was purchased from Kanto
Chemical Co., Inc. (Japan). The crude lipids from porcine brain were purified in our laboratory. High–performance TLC (HPTLC) glass plates were purchased from Millipore (Germany), whereas PVDF membranes, polytetrafluoroethylene (PTFE) membrane, and glass fiber filters were purchased from ATTO (Japan). Electric iron was purchased from Panasonic (Japan).

**Removing backgrounds from HPTLC plate**

The HPTLC plate was dipped in 1,2-dichloroethane (DCE) for 1 min, and dried in cold air for 10 min.

**Detecting lipids by coloring reagent**

After development, the imaging of total lipids was detected at 365 nm ultraviolet using a ChemiDoc system (Bio-Rad, USA) after spraying with primuline reagent, or glycolipids were visualized by heating the TLC plate at 120°C after spraying with orcinol–sulfuric reagent. The primuline reagent would slightly suppress ionization effect of glycosphingolipids, therefore we did not use the primuline reagent for the MSI analysis.

**TLC–blotting MSI**

Lipids were developed on HPTLC glass plates in chloroform/methanol/0.2% calcium chloride aqueous solution (Solution A, C/M/0.2% CaCl₂ aq., 60/40/9, v/v/v). After development, the HPTLC plate was dipped in 2-propanol/0.2% CaCl₂ aq./M (40/20/7, v/v/v) for 10 sec; thereafter the PVDF membrane, PTFE membrane, and glass fiber filter were piled up onto the HPTLC plate. The lipids were transferred by heating/pressing using an electric iron at 180°C for 30 sec. DHB was used as the matrix,
which was prepared at 5 mg/mL in 70% methanol. The PVDF membrane was pasted onto a MALDI target plate using an electric conductive tape. Finally, MALDI–TOF MS spectra of the lipids on the PVDF membrane were analyzed using a JMS-S3000 (JEOL, Japan) in positive ion mode, and the MSI was reconstructed using BioMap software (Fig. 1A-D, G, H). We used bradykinin fragment 1-7 (m/z 757.85) angiotensin II (m/z 1046.54), and adrenocorticotropic hormone fragment 18-39 (m/z 2465.20) for the calibration in all MSI analyses.

**TLC-MSI of Triglycerol**

To remove the background in the HPTLC plate with an aluminum backing, the HPTLC plate was soaked in DCE and developed in C/M (6:1, v/v) without any samples. After drying, Triglyceride (TG), diglyceride (DG), and monoglyceride (MG) dissolved in C/M (2:1, v/v) were applied to the aforementioned HPTLC. Lipids were developed in toluene/C/acetone (7:2:1, v/v/v). The developed HPTLC plate was visualized using copper sulfate reagent to confirm the Rf value. After development, another HPTLC plate was soaked for 5 sec in carbonate buffer at 85 mM and sodium acetate at 250 mM in a 1:1 ratio (CBS buffer), and dried in cold air for 10 min. Gold layers were deposited on the HPTLC at 35 mA for 36 sec using an MSP-1S sputter coater (Vacuum Device Inc., Japan). The HPTLC plate was placed on a MALDI–target plate with aluminum tape and analyzed by JMS–S3000 in positive ion mode and MSI was reconstructed using BioMap software (Fig. 1A, B, E-H).

**Results and Discussion**
**TLC-blot MSI analyses in various quantities of GlcCer**

To confirm the transfer and detection efficiency of the TLC-blot MSI, we analyzed various quantities of GlcCer (2, 4, and 8 µg/lane). The peaks derived from the molecular species in GlcCer were detected (Fig. 1A), and the detected peaks were assigned in Table 1. These molecular species in GlcCer were separated in slightly different positions of the band (data not shown). The reconstructed bands derived from the major peak at m/z 820 were increased in dose-dependent manner in the MSIs (Fig. 2B). However, background peaks were also detected in the MS spectra, only faint MSIs were obtained in small amounts of lipids (data not shown). To address this problem, we attempted to remove the background peaks by further optimizing the procedure.

**Removing of background peak from fixing agent of silica gel**

Next, we obtained the TLC-blot MSI of crude lipids purified from the porcine brain. Although some major lipids were detected, only minor lipids could not be detected owing to the background peaks (Fig. 3). In the accumulated MS spectra in the analyzed entire area, the background peaks were detected with high intensity in the entire area of TLC–blot MSI analysis (Fig. 3A). The background peaks of ion pattern were observed at 44 Da intervals (Fig. 3A). Generally, calcium sulfate (gypsum), starch and synthetic polymers are used as binders in TLC plates; therefore, they are transferred to the PVDF membrane together as we described before. To suppress the background peaks in TLC–blot MSI, the removal of binders on the TLC plate would be required before blotting. In a previous study, we confirmed that DCE could remove the background from glycolipids scraped and recovered from the TLC plate. We applied this method to remove the background from the TLC plate. Consequently, background peaks were suppressed by DCE pre–washing of the TLC plate, and minor lipids were detected (Fig.
3C). Furthermore, the band intensities derived from lipids after reconstruction in the TLC–blot MSI were increased (Fig. 3D). The detected major peaks were assigned in Table 2. These results demonstrate that DCE pre–washing of TLC plates improves the relative detection of lipids in TLC–blot MSI. Furthermore, the peak at $m/z$ 1278 was detected in different several bands in the MS imaging data (Fig. 3D). Therefore, GM1, GD1a, GT1a, and GT1b in the porcine brain was included the asialo-GM1 backbone (Fig. 3D).

Removing of backgrounds from TG for TLC-MSI

The common procedure of TLC-blot MSI for glycerin fatty acid esters (FAEs) is often difficult to apply, because their ionization efficiencies are poor using common matrices, such as DHB. Recently, the ionization method of FAEs has been developed, in which deposition of sodium salts and sputtering layer of gold on organ sections improve their ionization efficiency on tissue sections. We confirmed the separation of FAEs on HPTLC after visualization by copper sulfate reagent, FAEs (TG, 1,2-DG, 1,3-DG, or MG) were completely separated on the HPTLC (Fig. 4A). We analyzed the FAEs using TLC–blot MSI after the deposition of sodium salts and a sputtering layer of gold. However, no peaks were detected derived from FAEs in the MS spectra and no bands were observed in the reconstructed MSIs (data not shown). Therefore, we attempted to analyze FAEs separated on TLC directly for MSI analysis without blotting to the PVDF membrane. As shown in Fig. 4A, the peaks derived from TG were detected at $m/z$ 908; however, the relative intensities of the background peaks were higher than the TG peak (Fig. 4B). In addition, the reconstructed band derived from TG peak at $m/z$ 908 was faint (Fig. 4C). After DCE washing and C/M (6:1, v/v) HPTLC plates prior to development, the relative intensity of the TG peak detected at $m/z$ 908 was drastically
improved, and the band was changed to clear (Fig. 4D and E). We also confirmed that 1,2- and 1,3-DG were also clearly detected in the MS spectra and these bands were obtained as clear bands (data not shown). Although peak intensity was not detected with FAEs, TLC–MSI data of MG was also improved using the method developed (data not shown).

Recently, various ionization methods have been developed for lipid analysis using MSI, such as ozone-induced dissociation, nano–particle–assisted laser desorption/ionization (nano–PALDI), and secondary ion MS (SIMS)31-34. Although these techniques are quite powerful methods for lipid analyses, these methods often require extensive reagents, sample preparation/purification, or special instruments. We applied DCE pre-washing for background removal in a common HPTLC plate. The ionization efficiencies of lipids were drastically improved in TLC–MSI and TLC–blot MSI analyses using common matrix or gold sputtering. Therefore, these methods are thought to be easily applied to a wide research area. Other matrices such as 9-aminoacridine (9-AA), 1,5-diaminoaphthale (DAN), or dihydroxyacetophenone (DHAP) were also reported for specific lipid ionizations35-37. In the near future, we also apply these matrices for the established method.

**Conclusions**

In the present study, we demonstrated that DCE pre–washing of HPTLC plates can remove background effectively, and the ionization efficiencies of crude lipids in TLC–blot MSI or FAEs in TLC–MS after doping with sodium salts and gold sputtering were drastically improved. Consequently, the detected bands in the MSI also become clear. The obtained MSI data provide us the information of lipid compositions (FAEs) as well as head groups even in the faint bands derived from glycosphingolipids as the
slight different Rf values. In conclusion, the established method is particularly useful for minor lipid analysis using TLC (–blot) MSI. The current insufficient ionization efficiencies of MG or other low molecular weight lipids in TLC–MSI are the subject of a future study.

**Conflicts of interest**

The author(s) confirmed that there is no conflict of interest.

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Table 1  Assignment of the detected major peaks of GlcCer in MS spectra.

| m/z  | Ceramide structure (GlcCer)       | m/z  | Ceramide structure (GlcCer)       |
|------|----------------------------------|------|----------------------------------|
| 722  | [d18:1/C16:0 + Na]^+             | 794  | [d18:1/C20:0h + Na]^+            |
| 736  | [d18:1/C16:1h + Na]^+            | 804  | [d18:1/C22:1 + Na]^+             |
| 738  | [d18:1/C16:0h + Na]^+            | 806  | [d18:1/C22:0 + Na]^+             |
| 748  | [d18:1/C18:1 + Na]^+             | 820  | [d18:1/C22:1h + Na]^+            |
| 750  | [d18:1/C18:0 + Na]^+             | 822  | [d18:1/C22:0h + Na]^+            |
| 764  | [d18:1/C18:1h + Na]^+            | 832  | [d18:1/C24:1 + Na]^+             |
| 766  | [d18:1/C18:0h + Na]^+            | 834  | [d18:1/C24:0 + Na]^+             |
| 776  | [d18:1/C20:1 + Na]^+             | 848  | [d18:1/C24:1h + Na]^+            |
| 778  | [d18:1/C20:0 + Na]^+             | 850  | [d18:1/C24:0h + Na]^+            |
| 792  | [d18:1/C20:1h + Na]^+            |      |                                  |
Table 2  Assignment of the detected major peaks of crude lipids in porcine brain in MS spectra.

| m/z  | lipid composition          | lipid composition          |
|------|----------------------------|----------------------------|
| 766  | phosphatidylethanolamine   | [C36:2 + Na]⁺              |
| 772  | unknown                    | unknown                    |
| 810  | Phosphatidylcholine        | [C36:1 + Na]⁺              |
| 832  | GalCer                     | [d18:1/C24:1 + Na]⁺        |
| 847  | Phosphatidylserine         | [C40:0 + Na]⁺              |
| 848  | GalCer                     | [d18:1/C24:1h + Na]⁺       |
| 934  | unknown                    | unknown                    |
| 952  | Sulfatide                  | [d18:1/C24:0h + Na]⁺       |
| 1278 | asialo-GM1 (GM1-NeuAc)     | [d18:1/C18:0 + Na]⁺        |
Figure Captions

Fig. 1  Procedure of conventional TLC-blot MSI (B-D, G, H), developed TLC-blot MSI (A-D, G, H), or TLC-MSI using gold sputtering (A, B, E-H). Soaking HPTLC plate with DCE for removal of backgrounds (A), developing the lipids on HPTLC plate (B), transfer the separated lipids on PVDF membrane (C), spraying DHB solution on the PVDF membrane(D), gold-sputtering on HPTLC plate (E), soaking the HPTLC plate in CBS buffer (F), analysis of lipids by MALDI-TOF/MS (G), and reconstruction the MSIs of lipid distributions on HPTLC plate by BioMap software (H).

Fig. 2  Quantification of GlcCer using TLC-blot MSI. Total MS spectrum of GlcCer (A), and MSI data reconstructed from peak at m/z 820 by BioMap software (B). Arrow head indicates major peak derived from GlcCer.

Fig. 3  TLC-blot MSI analyses of crude lipids in porcine brain. Accumulated MS spectrum of separated lipids on PVDF membrane without DCE washing (A) and reconstructed MSIs of detected major peaks (B), or accumulated MS spectrum with DCE washing (B) and reconstructed MSIs of detected major peaks. Asterisk was indicated background peaks derived from HPTLC plate.

Fig. 4  TLC-MSI analysis of triglyceride after gold-sputtering. HPTLC analysis of FAEs visualized by copper sulfate reagent (A). Accumulated TLC-MS spectrum of triglycerol without DCE washing (B), and reconstructed TLC-MSI at m/z 908 (C). Accumulated TLC-MS spectrum of triglycerol with DCE washing (D), and reconstructed TLC-MSI at m/z 908 (E).
Fig. 1
Fig. 2
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