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A new lipidomics approach by thin-layer chromatography-blot-matrix-assisted laser desorption/ionization imaging mass spectrometry for analyzing detailed patterns of phospholipid molecular species

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ABSTRACT

Thin-layer chromatography (TLC) is a highly established convenient technique for lipid separation and partial characterization of neutral and acidic glycosphingolipids (GSLs) and phospholipids, in mixtures. Meanwhile, imaging mass spectrometry (IMS) is a promising tool for lipidomics. However, some lipid classes are detected more sensitively than others, which can lead to suppression effects when complex mixtures are analyzed. Therefore to analyze complex lipid mixtures, a precise separation into the individual lipid classes is necessary. Here we present our highly sensitive and convenient analytical technology that combines TLC and IMS, namely the TLC-Blot–MALDI-IMS method, to visualize whole lipids and individual molecular species with high sensitivity compared with common staining methods. This method allows for easy visualization of all lipids with a linear range of approximately one order of magnitude and precision <16% RSD, making it useful for differential display analysis of lipids.

1. Introduction

Many different methods for lipid analysis have been established, including chromatographic (HPLC or high-performance thin-layer chromatography (HPTLC)), spectroscopic (e.g. NMR), and mass spectrometric methods [1]. Due to its high sensitivity, mass spectrometry (MS) is regarded as one of the most powerful methods of lipid analysis [2], and the term "lipidomics" has recently been introduced [3]. Using soft ionization techniques, nearly all lipid classes can be analyzed very sensitively without major fragmentation. Although electrospray ionization [4,5] and atmospheric pressure chemical ionization [6] are thus far the most widely used methods in the lipid field, there is growing evidence that matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF) MS represents a potential alternative [7–9] MALDI-TOF MS analyses can be performed quickly with high sensitivity and can ionize lipids even in the presence of contaminants. The introduction of samples into the MS instrument is simple in MALDI-MS [10–12]. Recently, there has been an increasing number of studies on imaging mass spectrometry (IMS), which is one of the applications of MALDI-TOF MS [13–17]. In these studies, some classes of lipids have mainly been detected in frozen tissue. IMS that utilizes MALDI technology provides a molecular *ex vivo* view of dissected organs or animal whole-body sections, making possible the label-free tracking of both endogenous and exogenous compounds with sufficient spatial resolution and molecular specificity of targets [16–22]. The most useful conventional lipid detection methods have been immunoassays and staining methods, but these methods just recognize a part of the structure. Thus, IMS is a revolutionary method for detecting lipids by their mass [23–28]. In IMS, however, the ability to detect the individual lipid classes differs significantly [29]. It has been shown that the presence of lipids with quaternary amines, as in phosphatidylcholine (PC), lyso-phosphatidylcholine (LPC), and sphingomyelin (SM), tends to suppress other lipid ionization [30]. It is therefore very difficult to visualize by IMS other lipid classes such as phosphatidylethanolamine (PE) and phosphatidylserine (PS) in tissue specimens. Although this problem can be partially overcome by comparing the negative and positive ion mode spectra or by using less acidic matrix compounds such as THA [31] or PNA [32], it is important that complex lipid mixtures be separated into the individual lipid classes for lipidomics research.

Thin-layer chromatography (TLC) is a highly established, inexpensive, and convenient technique for lipid separation [33–35]. However, even under optimized TLC conditions, TLC does not yield unambiguous structural information for individual phospholipids (PLs). The TLC profile visualized by chemical staining such as with molybdenum blue for phospholipids and orcinol for gly-

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colipids informs us of the separation based on the hydrophilic moieties. However, the hydrophobic properties of individual lipid classes also influence the mobility on the HPTLC plate. For example, galactosylceramide (GalCer) and sulfatide from brain tissue give double bands based on the different hydrophobicities between ceramide containing normal fatty acids and those containing hydroxy-fatty acids. This suggests that phospholipid molecular species based on hydrophobic moieties can be analyzed if sensitive IMS analysis is employed for the separated bands on the HPTLC plate.

Direct TLC–MS presents difficulties with regard to complete retrieval of the target compound from the silica coated on the TLC plate and can also cause the MS instrument to be contaminated with silica. Transfer methods for proteins have also been reported and used widely [36,37]. As for the transfer of lipids from TLC plate, Towbin et al. first demonstrated a procedure using nitrocellulose [38]. However the efficiency of the transfer was unsatisfactory. We, however, have found that this TLC-Blot can be carried out using PVDF membrane and heating system [39], then established our method [40,41]. The transferred lipids remain stable and samples are concentrated on one side on the PVDF membrane. These difficulties of direct TLC–MS have largely been resolved with a method that we introduced previously, in which the separated lipids are transferred from a high-performance TLC (HPTLC) plate onto a PVDF membrane. The PVDF membrane is attached to a MALDI target plate and analyzed by TOF. We have designated this method as TLC-Blot–MALDI-MS [42]. We consider that TLC-Blot–MALDI-MS is better than TLC–MALDI-MS for analysis of minor components in small amounts of biologically complex mixtures.

In the present study, we investigated TLC-Blot–MALDI-IMS analyses with a Nd:YAG laser and a standard 2,5-dihydroxy benzoic acid (DHB) matrix to scan transferred PVDF membrane to detect each PL and molecular species by MS analyses. The two-dimensional scanning enabled us to visualize all transferred lipids. Here we present details regarding the TLC-Blot–MALDI-MS method combined with IMS technology to simultaneously visualize whole lipids as well as each molecular species with high sensitivity, with possible application to differential display analyses.

2. Experimental

2.1. Chemicals and materials

L-α-Dipalmitoyl-phosphatidylcholine was purchased from Wako Pure Chemical Industries (Osaka, Japan). All solvents used for MS were of HPLC grade and were purchased from Kanto Chemical Co., Inc. (Tokyo, Japan). Bradykinin and angiotensin-II were obtained from Sigma–Aldrich Japan (Tokyo, Japan) and used as calibration standards. 2,5-Dihydroxy benzoic acid (DHB) obtained from Bruker Daltonics (Leipzig, Germany) was used as the matrix.

The brain tissues were obtained from the Netherlands Brain Bank (NBB). All samples were anonymous and were obtained under conditions of informed consent according to the ethical guidelines of the NBB. Tissue (approximately 0.1 g) was homogenized with twenty fold volume of chloroform/methanol (2/1, v/v) and sonicated for 5 min.

2.2. Thin-layer chromatography

Phospholipids dissolved in 100% methanol and human total lipid extracts dissolved in chloroform/methanol (2/1, v/v) were applied as 5-mm width spots to silica gel 60 HPTLC plates (Merck, Darmstadt, Germany) manually. Plates were developed with a solvent system of methylacetate/propanol/chloroform/methanol/0.25% aqueous KCl (25/25/25/10/9, v/v/v/v). After the development of samples, the HPTLC plate was sprayed with primuline reagent until the plate became wet and then was air-dried thoroughly. Lipid bands were visualized under UV light at 366 nm. The visualized bands are marked with soft pencil under UV light.

2.3. TLC-Blot

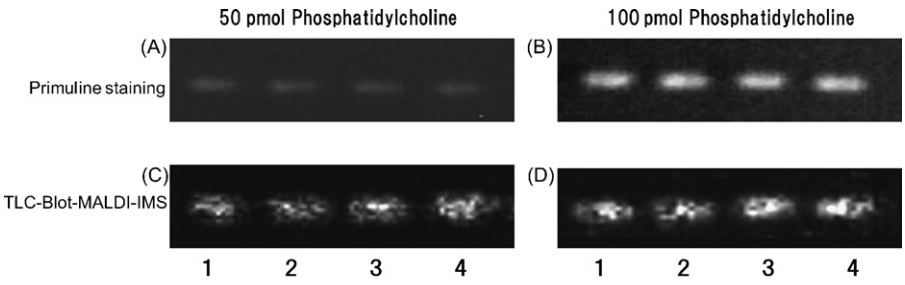
The TLC-Blot was performed as described previously [39]. Briefly, the HPTLC plate was dipped in the blotting solvent (2-propanol/0.2% aqueous $CaCl_2$/methanol, 40/20/7, v/v/v) for 10 s, after which the HPTLC plate was immediately placed on a flat glass plate. First, a PVDF membrane, a Teflon membrane, and then a glass fiber filter sheet were placed over the plate. This assembly was pressed evenly for 30 s with a thermal blotter (ATTO, AC-5970, Tokyo) at 180 °C. The PVDF membrane, removed from the HPTLC plate, was then air-dried. Pencil marks were transferred to the PVDF membrane facing the HPTLC plate, but lipids were exclusively located on the reverse side.

2.4. TLC-Blot–MALDI-TOF MS

MALDI-TOF MS analyses were performed using a MALDI-hybrid quadrupole TOF-type mass spectrometer (QSTAR XL, Applied Biosystems/MDS Sciex, Foster City, CA) equipped with an orthogonal MALDI source and a Nd:YAG laser at a repetition rate of 100 Hz. Samples were analyzed in positive ionization mode over the range of m/z 100–2000. The MS spectra were calibrated externally using a standard peptide calibration mixture containing 10 pmol/μl each of bradykinin peptide fragment (amino acid residue 1–7) ($[M+H]^+$, m/z 757.40) and human angiotensin-II peptide fragment ($[M+H]^+$, m/z 1046.54). A methanol/0.1% TFA solution (1/1, v/v) containing 50 mg/ml DHB was used as the matrix. The PVDF membrane was attached to a MALDI sample plate with electrified double-adhesive tape all over to reduce charge-up of the plate. Sufficient amounts of DHB solution (totaling 100–150 μl) were then manually deposited to the PVDF membrane, which was transferred by the heat iron method from the TLC plates. The crystallization process was accelerated under a gentle stream of cold air. Moreover, pressing the PVDF membrane to contact the electrified conductive tapes was important for achieving good mass spectra.

2.5. Imaging mass spectrometry

The imaging mass spectrometry scan of the PVDF membrane was performed automatically. MALDI-IMS spectra were obtained from a Nd:YAG laser at a repetition rate of 100 Hz per image spot with a 1 s accumulation time. The instrument is equipped with oMALDI Server application. As the profiles are being acquired, the sample stage is moved from spot to spot, creating a raster of desorbed areas over the tissue surface. A spectrum is acquired at each spot, and the intensity of each signal from all of the mass ranges specified is stored in a "wiff" format file. When the "wiff" format file was converted to the "img" format file using the function of oMALDI Server ver. 5.1, the interval value of m/z was set to 0.2. The ion density maps were constructed using BioMap software (Novartis, Basel, Switzerland) and highlighted the relative amounts of signal intensity obtained from regions of interest within the PVDF membrane. The following experimental parameters were used for imaging: scan pitch, 400 μm; scan area, 3 cm × 3 cm. The scanning time took 1.56 s per spot. The overall scanning time was 2.4 h. Statistical analyses were performed by SPSS statistics 17.0 (SPSS Japan Inc., Tokyo, Japan).



(E)

method	concentration of analyte pmol	analysis results(n=4) RSD(%)
Primuline staining	50	11
Imaging mass spectrometry	50	15.7 (17.8)
Primuline staining	100	7.8
Imaging mass spectrometry	100	9.4 (11.9)

Fig. 1. The optical images of primuline staining of phosphatidylcholine (PC) 50 pmol (A) and 100 pmol (B), the ion images of TLC-Blot–MALDI-IMS of PC (m/z 756.54) of 50 pmol (C) and 100 pmol (D). The RSD values of each intra-plate measurement (inter-plate) are also shown (E).

3. Results and discussion

3.1. The detection limit of phospholipids by TLC-Blot–MALDI-TOF MS

There have been some reports of TLC-MALDI regarding phospholipids. Rohlfing et al. used an infrared (IR) laser in combination with an orthogonal MS device [43]. In addition, Fuchs et al. have shown that analysis of phospholipids from a TLC plate is also possible using axial MALDI-TOF MS [44,45] with a UV laser and a standard DHB matrix. However, these studies were only performed with relatively simple PL mixtures, and there was no discussion of the reproducible and quantitative aspects of their method. We first tried to validate the quantitative and reproducible transfer of lipids from an HPTLC plate to the PVDF membrane. We developed four lanes of the same amounts of L-α-dipalmitoylphosphatidylcholine (M.W. 733) by TLC. We then detected and visualized these molecules by the conventional staining method (Fig. 1A and B). For the optical images of primuline staining, there was reproducibility at both 50 and 100 pmol. Previous reports have shown that the results of the primuline staining method are reproducible [46–48]. Our results also showed that the values of relative standard deviation (RSD) are sufficient to show reproducibility, that is, 11% (50 pmol) and 7.8% (100 pmol). Next, we tried to detect same samples by TLC-Blot–MALDI-IMS. In the MS spectrum, PCs were detected primarily as sodium-adduct ion $[M+Na]^+$. The ion images at m/z 756.5 were constructed, and the density was measured by Image J software (Fig. 1C and D). The RSD values of TLC-Blot–MALDI-IMS were high compared with those obtained with primuline staining, but the intra-plate precision was sufficient for semi-quantitative analyses, that is, 15.7% (50 pmol) and 9.4% (100 pmol) (Fig. 1E). We also calculated the inter-plate RSD of the TLC-Blot–MALDI-IMS method measured on another day, which resulted in values of 17.8% (50 pmol) and 11.9% (100 pmol). These results indicated that the new method, TLC-Blot–MALDI-IMS, is comparatively reproducible.

Next, we investigated the limit of detection (LOD) by using variable concentrations of PCs. The PCs were applied to the TLC plate at quantities of 5, 10, 20, 50, and 100 pmol PCs and were developed with the conventional solvent. Fig. 2A shows an optical image of the TLC plate stained by primuline. The band corresponding to 20 pmol is barely visible, and that corresponding to 10 pmol of PCs can hardly be seen. The LOD of primuline staining can therefore be considered to be 20 pmol. The lipids on the developed TLC plate were then transferred to a PVDF membrane following the methods described by the authors [42]. The membrane was fully laser-scanned by the IMS method, and the ion image of m/z 756.5 was constructed (Fig. 2B). The band corresponding to 10 pmol of PCs

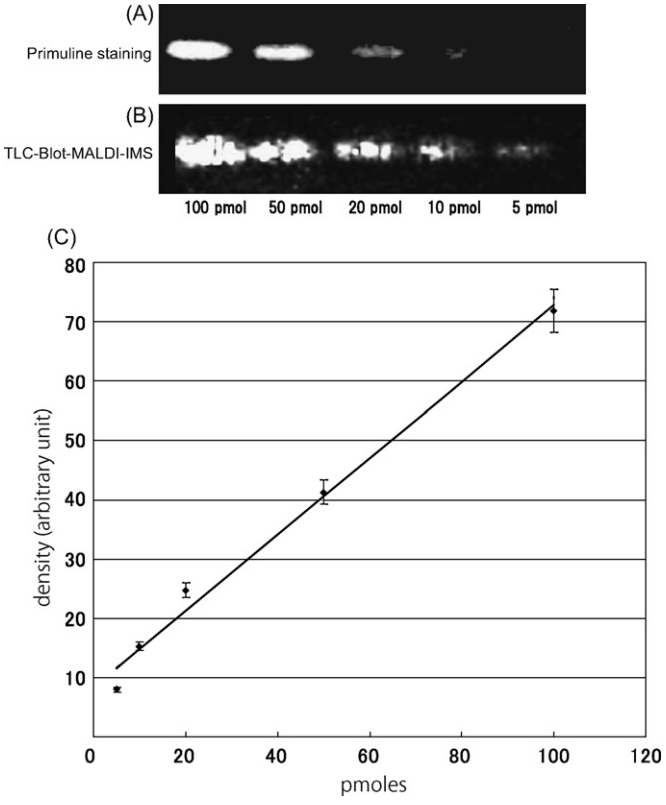


Fig. 2. (A) Thin-layer chromatogram stained with primuline. Lanes (1–5) contain PC: (lane 1) 100 pmol, (lane 2) 50 pmol, (lane 3) 20 pmol, (lane 4) 10 pmol, and (lane 5) 5 pmol. (B) The same PVDF membrane was directly analyzed by TLC-Blot–MALDI-IMS. (C) The calibration curve of the TLC-Blot–MALDI-IMS method was calculated.

could be seen; furthermore, the band corresponding to 5 pmol was detectable, though it was a low signal. Our results show that the LOD of TLC-Blot–MALDI-IMS is as low as 5 pmol PC. Moreover, the calibration curve was made according to these ion intensities, and the value of the determination coefficient showed sufficient linearity (R^2 = 0.9896). This method might make it possible to detect minor components of lipids that are not detected by primuline staining and it can also be applied to differential analyses of various lipids due to its quantitative potential and ability to simultaneous assay of multiple lipid variances.

3.2. Imaging mass spectrometry analyses of human brain phospholipids

We next applied this method to total lipids extracted from human brain samples. One previous report has described direct MALDI-MS of brain extracted lipids [49]. We, however, first collected the lipids from four different sites, white and gray matter of both the inferior frontal gyrus and the hippocampus. Using this new method, we tried to demonstrate the differential display of phospholipids among the different positions of the brain and to identify lipids having a specific distribution. Total lipids that were extracted by the conventional method were developed by TLC and stained by primuline (Fig. 3A). There were several bands, and they were annotated as galactosylceramide, sulfatide, phosphatidylethanolamine, phosphatidylinositol (PI), phosphatidylserine, phosphatidylcholine, and sphingomyelin by their positions relative to the front value and our study (paper will be published elsewhere). In white matter of the inferior frontal gyrus and hippocampus, GalCer, sulfatide, and SM, major components of myelin, were abundant. However, we could not perceive a difference in the amounts of other lipids among the samples. By the primuline staining procedure, PE, PS, PC, and SM were detected as respective single broad bands, that is, the lipids were separated depending on the properties of their hydrophilic moieties. Fig. 3B and C show MS spectra of the PC and SM bands, respectively. There are various peaks annotated as PC and SM. In the MS spectrum of PC, major peaks such as m/z 810.5, 782.5, 760.5, 734.5, and 723.4 were observed. It is clear that these molecules were PC because their ion intensity was high on the band of PC. It is well known that the linkage at the second position of glycerol is also an acyl linkage, but that the first position of glycerol is not only an acyl linkage but also is sometimes substituted with alkyl- or alkenyl linkages. Therefore, in this report we use the following nomenclature to specify the signals according to the system of Lipid Search (http://lipidsearch.jp/manual_search/): [phospholipid name (1-acyl total number of fatty acid carbons: number of double bond). Based on this rule, peaks were identified as [PC (1-acyl 36:1)+Na]$^+$, [PC (1-acyl 34:1)+Na]$^+$, [PC (1-acyl 34:1)+H]$^+$, [PC (1-acyl 32:0)+Na]$^+$, and [PC (1-acyl 34:1)+Na–N$(CH_3)_3$]$^+$, respectively, based on their mass and previous reports [17]. In addition, in the MS spectrum of SM, we observed m/z 835.6, 781.5, 753.5, 731.5,

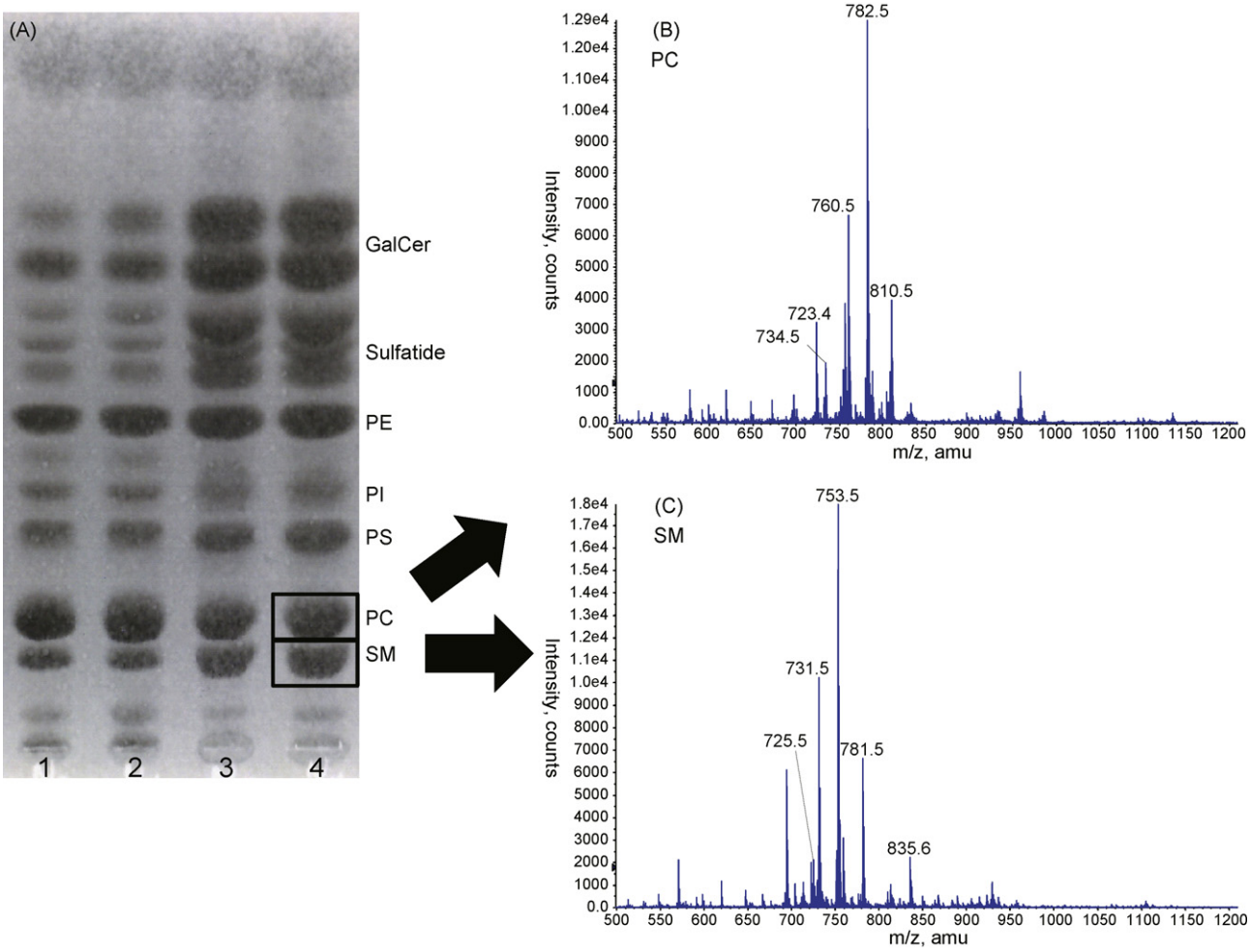


Fig. 3. (A) Thin-layer chromatogram stained with primuline. Lanes (1–4) contain human brain lipid extracted from gray matter of inferior frontal gyrus (lane 1), gray matter of hippocampus (lane 2), white matter of inferior frontal gyrus (lane 3), and white matter of hippocampus (lane 4). The MS spectra of PC (B) and SM (C) allowed us to directly analyze the blotted PVDF membrane.

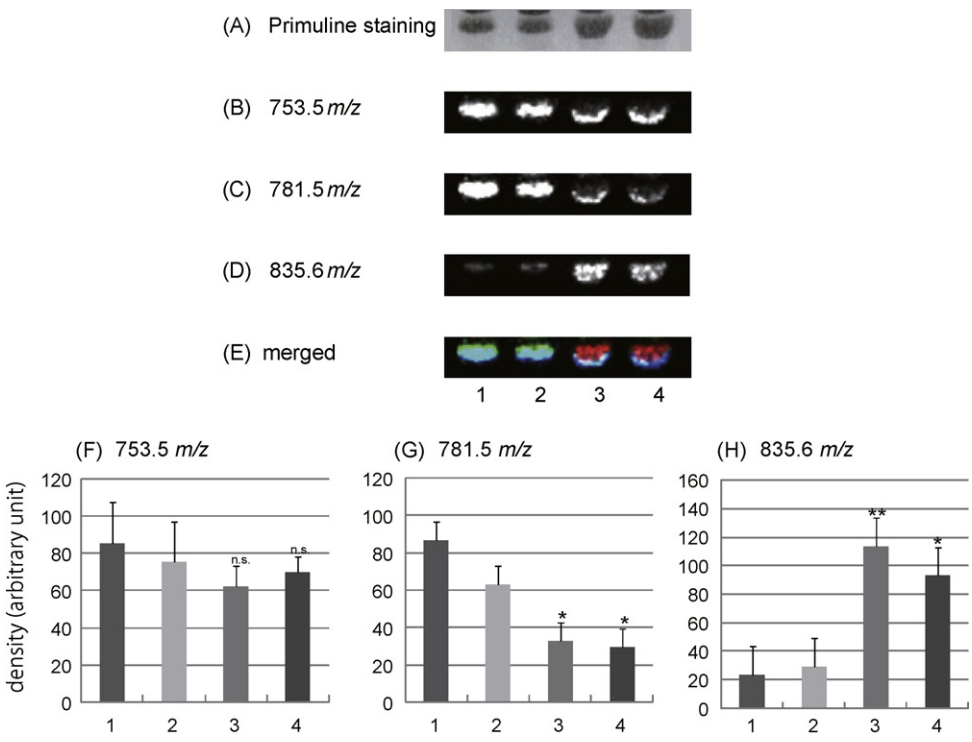


Fig. 4. The cut optical image of primuline staining (A), the ion images of *m*/*z* 753.5 (B), 781.5 (C), and 835.6 (D) are shown. The merged image of these three ion images is also shown (red, *m*/*z* 835.6; green, *m*/*z* 781.5; blue, *m*/*z* 753.5) (E). These ion intensities were quantified by the SPSS statistics (F, G, and H, respectively). Data are presented as the mean ± S.E. ($n = 3$). The lipid amounts of SM (d18:1/C20:0) and (d18:1/C24:1) differ significantly between gray and white matter levels at $^{**}p < 0.001$; $^{*}p < 0.05$; n.s., not significant. For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.

and 725.5 as major peaks. It is clear that these molecules were SM because their ion intensities were high on the band of SM. These molecules were assigned as [SM with d18:1 sphingosine and C24:1 fatty acid (d18:1/C24:1)+Na]$^+$, [SM (d18:1/C20:0)+Na]$^+$, [SM (d18:1/C18:0)+Na]$^+$, [SM (d18:1/C18:0)+H]$^+$, and [SM (d18:1/C16:0)+Na]$^+$, respectively, based on a previous report [50] and the use of Lipid Search (http://lipidsearch.jp/manual_search/). We have also detected mass spectra of GalCer, sulfatide, PS, and PE by the scanning of each band (data not shown). In the band of PI, we could not detect characteristic peaks because PI was usually detected in negative ion mode. Based on the TLC-Blot–MALDI-IMS, we could detect various kinds of molecular species and identify their structures. It is clear that GalCer, sulfatide, and SM were abundant in the white matter. It is not possible to determine what molecular species are characteristic in GalCer, sulfatide, and SM by the chemical staining detection method. In TLC-Blot–MALDI-IMS, however, we can construct individual ion images of their molecular weights, which enable us to visualize each molecular species on a TLC plate and thereby distinguish between the samples. We therefore tried to analyze SM of human brain lipid extracts (Fig. 4A) by TLC-Blot–MALDI-IMS and constructed ion images at *m*/*z* 753.5 (d18:1/C18:0), 781.5 (d18:1/C20:0), and 835.6 (d18:1/C24:1) (Fig. 4B–D). A merged image of these ions (blue; *m*/*z* 753.5 (d18:1/C18:0), green; *m*/*z* 781.5 (d18:1/C20:0), red; *m*/*z* 835.6 (d18:1/C24:1)) is also shown (Fig. 4E). We were able to find that these SM molecules were localized differently between the gray matter and white matter. The molecule at *m*/*z* 753.5 (d18:1/C18:0) was detected equivalently in all samples (Fig. 4F), but *m*/*z* 781.5 (d18:1/C20:0) was detected predominantly in gray matter (Fig. 4G). In contrast, the molecule at *m*/*z* 835.6 (d18:1/C24:1) was detected in the white matter (Fig. 4H). We repeated the analyses ($n = 3$), measured the ion intensities, and performed statistical analyses. The results showed that the amounts of SM (d18:1/C20:0) and (d18:1/C24:1) differed significantly between the gray and white matter. In the optical image, we could see that all SM molecules were abundant in the white matter compared with the gray matter. We were able to identify a region-specific distribution of individual lipids in terms of their molecular species by the use of TLC-Blot–MALDI-IMS.

4. Conclusion

We have described here a new procedure for highly sensitive and semi-quantitative detection analysis of PLs as a visual display by TLC-Blot–MALDI-IMS. We confirmed that our method is suitable for differential display of molecular species of a particular lipid among different sources such as gray matter and white matter of the brain region. We also recognize the problem of comparing amounts between different molecules because the ionization efficiency of molecules is dependent on their structures. But the whole amount of the individual lipids can be estimated by densitometric analysis of the TLC profile. This procedure is very easy to carry out and requires no specific equipment or antibodies to detect and discriminate between analytes; usually, only one-time TLC and transfer to a PVDF membrane make it possible to separate, visualize, semi-quantify and identify PLs at a low picomole level in half a day. In the present study, the TLC-Blot–MALDI-IMS to the PLs band successfully discriminated each molecular species and allowed us to compare these lipids with various samples. We believe that this method will in the future be useful for differential display of lipids.

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