Mapping Isomeric Cerebroside Distribution in Rat Brain via MSI with MALDI-TIMS and Signal Enhancement through On-Tissue Enzyme Treatment

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Introduction

Cerebrosides (CBs), the simplest glycosphingolipids, play vital roles in the brain. They consist of a monosaccharide—glucose or galactose—linked to ceramide via a β-glycosidic bond. Conventional MS struggles to distinguish diastereomeric CBs, masking their distinct biological roles. Moreover, galactosylceramides (GalCer) are the predominant species in mammalian central nervous systems, complicating the detection of the less abundant glucosylceramides (GlcCer). We developed a phospholipase C-assisted MALDI2-TIMS-TOF MSI approach employing offline enzymatic digestion to remove phospholipids, reducing potential ionization suppression. MALDI-2 post-ionization enhanced the ionization of cerebrosides, and TIMS was used to resolve CB isomers. Our findings uncover the distinct spatial distributions of GalCer and GlcCer in rat brains, underscoring the importance of differentiating diastereomeric CB pairs to elucidate their biological roles.

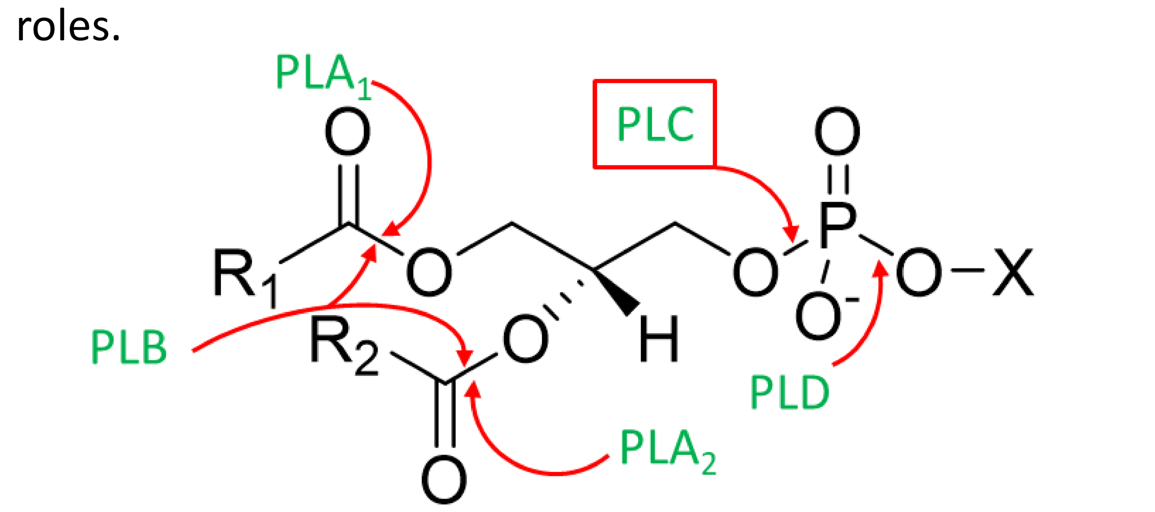


Figure 1. Different cleavage site from phospholipase

Materials and Method

All experiments were conducted on a timsTOF fleX MALDI-2 mass spectrometer (Bruker).

Rat brain sections (16 µm) were mounted on ITO-coated glass slides (Delta Technologies). Phospholipase C (50 U mL^{-1}, *Bacillus cereus*, Sigma) was evenly applied (50 µL) and samples were incubated at 37°C for 30 minutes. Afterward, specimen-surrounding solution was removed via vacuum suction, and tissues were washed with 50 µL of 50 mM ammonium bicarbonate for 5 minutes.

Sections were coated with 1,5-diaminonaphthalene (DAN, 20 mg mL^{-1} in ACN/H_2O 70:30) using an HTX-M5 Sprayer (HTX Technologies). Mass spectrometric analysis involving MS imaging (MSI) and ion mobility separation was performed using timsControl and FlexImaging software (Bruker), with data analyzed via DataAnalysis Compass,SCiLs Lab (Bruker), in-house R codes, and Metaboanalyst.

PLC-assisted MALDI-2 TIMS MSI Optimization

TIMS Optimization – Ion-type Selection/Ramp Time Optimization

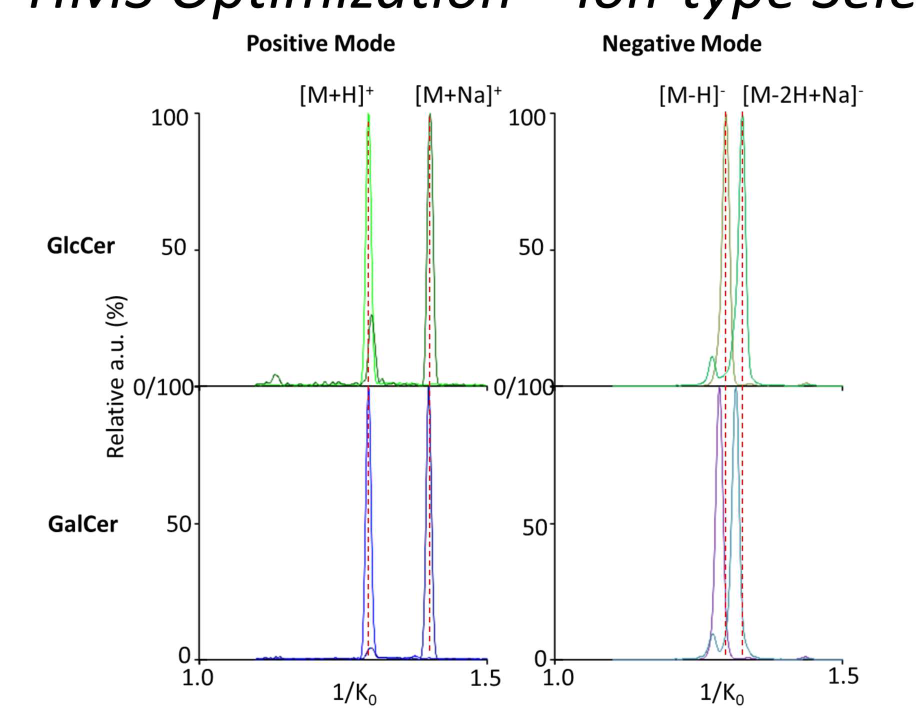


Figure 2. Ion-type selection for TIMS-based separation under default settings. Commonly observed cation species ([M+H]$^+$ and [M+Na]$^+$) do not exhibit mobility separation, whereas commonly observed anion species ([M−H]$^-$ and [M−2H+Na]$^-$) demonstrate clear ion mobility separation.

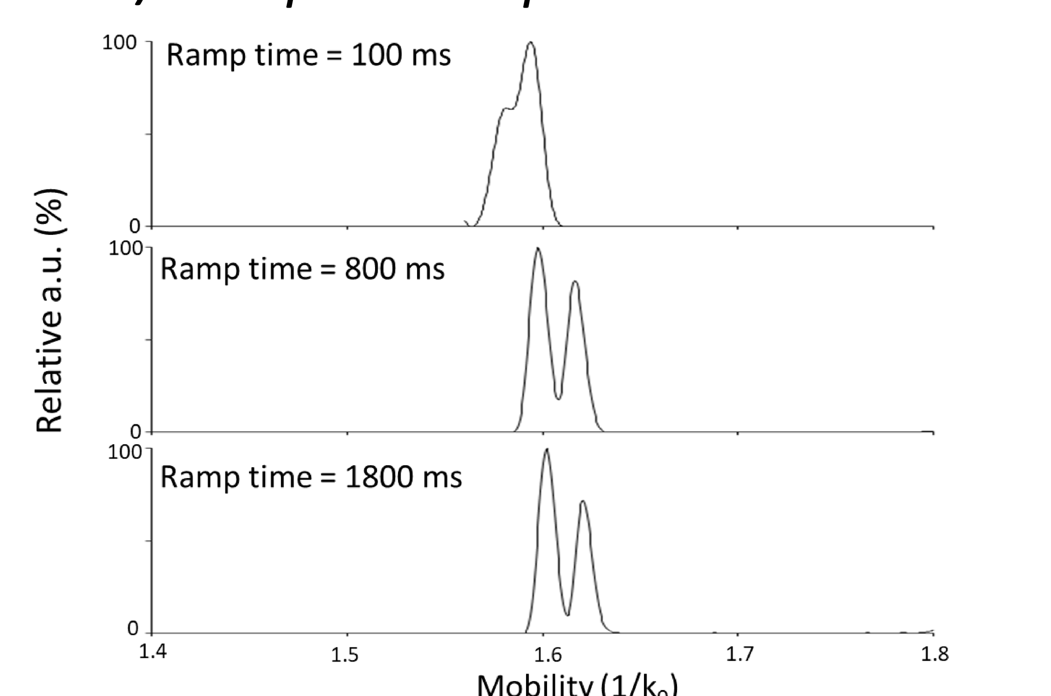
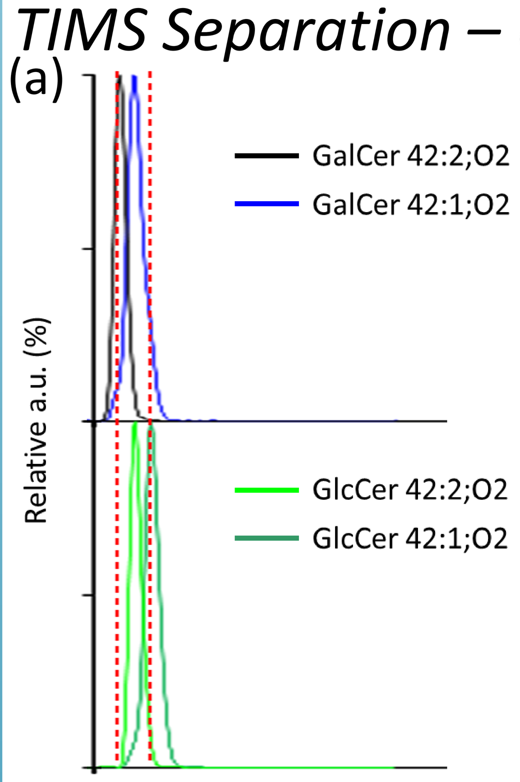


Figure 3. Optimization of TIMS settings for isomeric cerebroside separation using different ramp times. Extracted ion mobilograms (EIMs) of HexCer 34:1;O2 (m/z 689.558) at varying ramp durations demonstrate improved separation, with full width at half maximum (FWHM) resolution approximately doubling under optimized conditions.

TIMS Separation – Unsaturation Side Chain and α/β Glycosidic Linkage



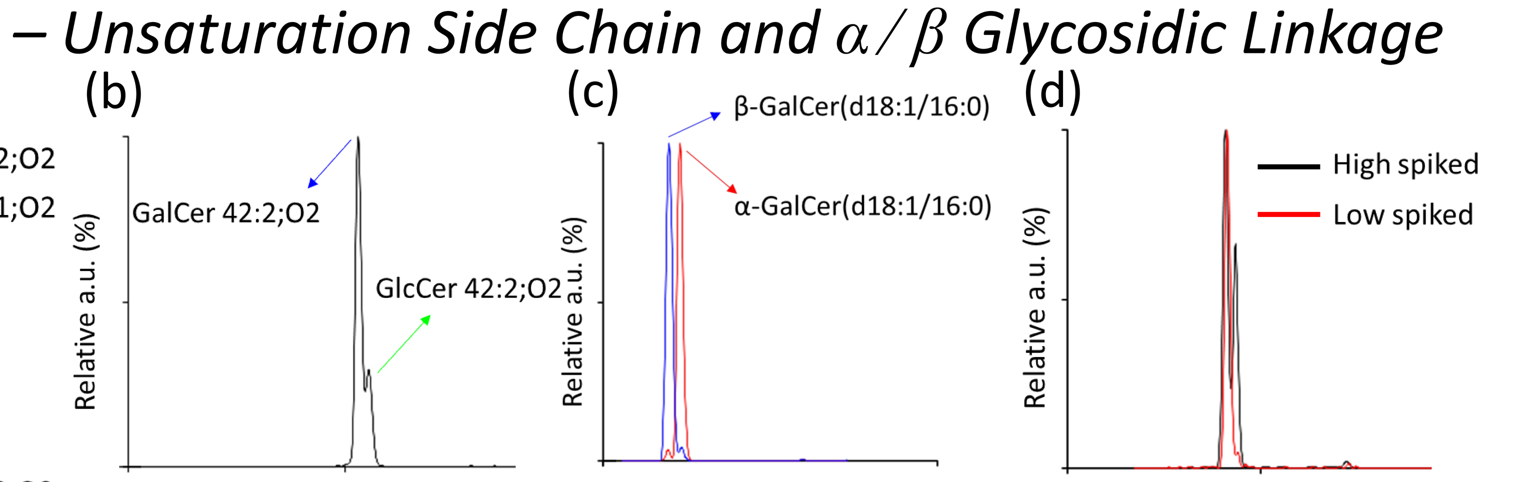


Figure 4. TIMS-based separation of isomeric cerebrosides. (a) Extracted ion mobilograms (EIMs) of HexCer 42:1;O2 (m/z 810.683) and HexCer 42:2;O2 (m/z 808.667) from brain cerebroside extracts and spiked standard sample; (b) EIM of GlcCer 42:2;O2 (m/z 808.667) spiked into a cerebroside extract matrix, confirming isomer separation in complex mixtures; (c) EIM of α- and β-anomers of GalCer (d18:1/16:0), illustrating their distinct ion mobility profiles; (d) Estimated percent difference in mobility between resolved isomers (~0.5%), demonstrating the platform's high resolving power.

PLC Sample Preparation with Laser Size Optimization

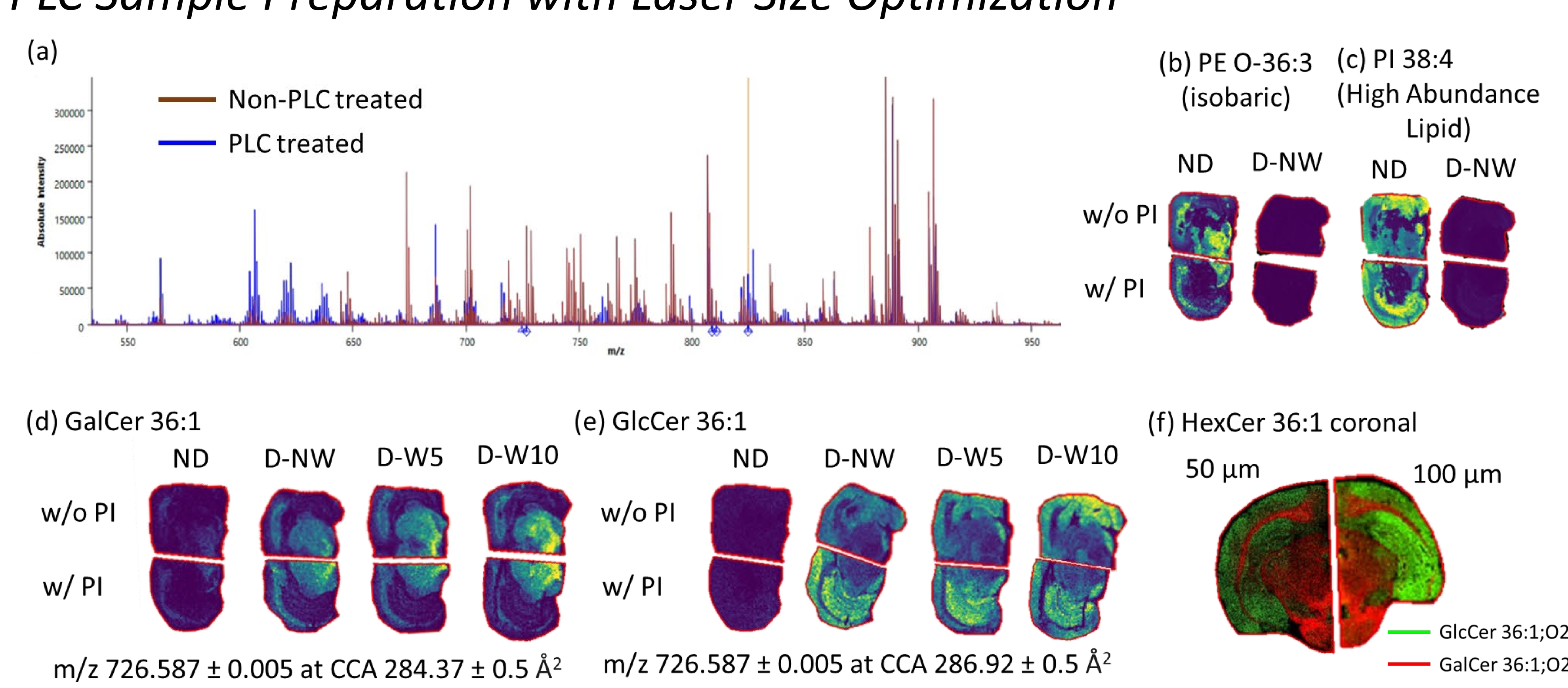
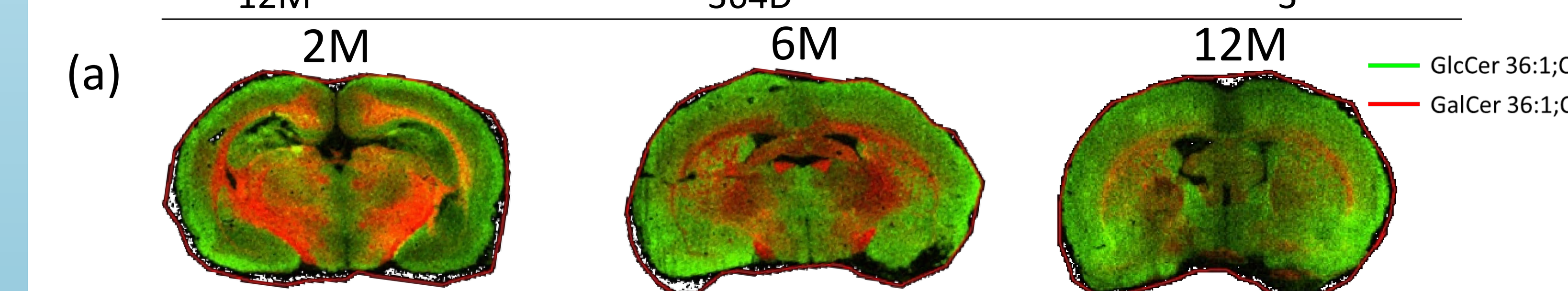


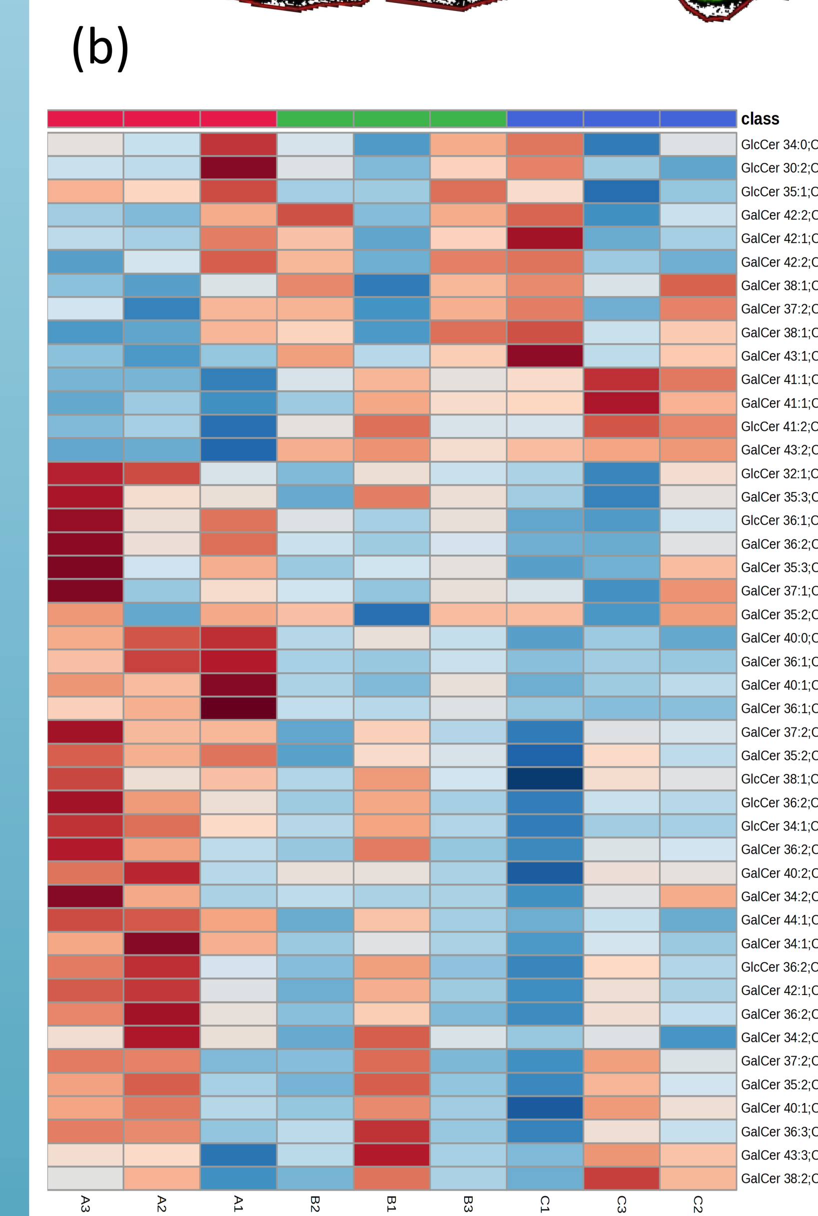
Figure 5. Optimization of PLC-Assisted MALDI-TIMS-TOF MSI for isomeric cerebrosides. (a) Comparison of MS spectra before and after PLC digestion. MSI comparisons of (b)PE O-36:3, an isobaric lipid, (c) PI 38:4, high abundance phospholipid, (d) GalCer 36:1, and (e) GlcCer 36:1 with and without post-ionization (PI) under different sample preparation strategies. **ND**: no PLC digestion; **D-NW**: PLC digestion without a washing step; **D-W5** and **D-W10**: PLC digestion followed by washing with 50 mM ammonium bicarbonate for 5 or 10 minutes, respectively. Overlaid MSI images of GalCer 36:1 and GlcCer 36:1 in (e) coronal brain sections. Laser beam sizes of 50 µm (left in f) and 100 µm (right in f.)

Simple Rat Aging Model Results

Rat age Table - Sprague-Dawley rat

Age group	Real Age (based on recorded DOB)	Total animal
2M	61D	3
6M	180D	3
12M	364D	3





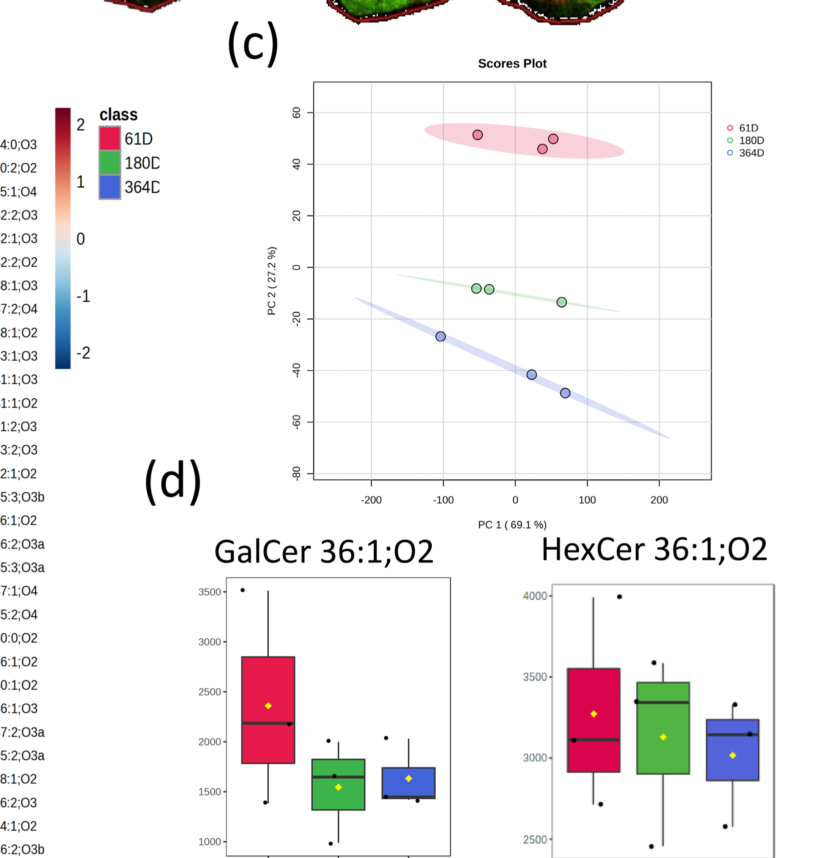


Figure 6. Age-dependent changes in cerebroside distribution and composition in rat brain. (a) Spatial distribution and age-related changes of representative cerebrosides across three age groups; (b) Heatmaps showing trends in total cerebroside profiles among rat brains; (c) PCA reveals clear group separation by age; (d) Comparison between isomer-resolved GalCer 36:1;O2 and pooled HexCer 36:1;O2 results demonstrates that resolving isomers provides more precise and biologically relevant comparisons.

Conclusions

This work successfully established a MSI platform that integrates on-tissue phospholipase C treatment TIMS to reveal the spatial distribution of isomeric cerebrosides in the rat brain. A total of 45 cerebroside species were profiled from rat aging model, and the results indicate that cerebroside levels change with age, suggesting a role in aging-related metabolic processes. Future directions include expanding the lipid coverage to encompass a broader range of glycolipids for deeper insights into isomeric glycolipid metabolism, and adapting this platform for single-cell analysis to enable detection of low-abundance isomeric glycolipids at cellular resolution.

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