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Resolving brain regions using nanostructure initiator mass spectrometry imaging

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Abstract

Specific cell types are critically implicated in a variety of neuropathologies that exhibit region-specific susceptibility. Neuronal and glial function is impaired in a host of neurodegenerative diseases. Previous reports suggest that mass spectrometry imaging has the potential to resolve cell-specific enrichment in brain regions; however, individual ions cannot resolve glial and neuronal cells within the complex structure of brain tissue. Here, we utilized a matrix-free surface mass spectrometry approach, nanostructure initiator mass spectrometry (NIMS) to determine if multiple lipid ions can be used as a ‘fingerprint’ to discriminate between neuronal- and glial-enriched brain regions and between glial cells in different regions of the brain, such as the cortex and the cerebellum. This is accomplished by using established stains to define glial cell enrichment (GFAP) and NIMS imaging on adjacent serial sections to measure phospholipid distributions. Identifications were made using QTOF analysis of whole-brain extracts and comparison with previous reports. From these identifications, it was found that utilization of multiple lipids can indeed resolve glial and neuronal cell enriched brain regions in the imaged brain section. The resolution of brain regions and cell populations is greatly enhanced through application of multivariate statistical analysis (Nonnegative Matrix Factorization) of the 18 dominant potassium adducts of phospholipids. Strikingly, this analysis resolves other brain regions that are difficult to distinguish using conventional stains but known to have distinct physiological functions. For example, this method could accurately distinguish the frontal (or somatomotor) and dorsal (or retrosplenial) regions of the cortex, which have important functional differences, from each other and from the pons region. This work suggests that using this approach with inclusion of larger numbers of lipids has the potential to greatly improve our understanding of regional and cell type specific variation in the brain.

INTRODUCTION

The central nervous system has the highest concentration of lipids, next to adipose tissue¹. Lipids play both structural and functional roles ranging from development of the neocortex to the processing of complex behaviors through neurotransmitters, neuropeptides, and growth factors². Consistent with these roles, differential expression of phospholipid molecules is strongly associated with the progression of neurodegenerative diseases,

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including Alzheimer and Huntington's Diseases^{3,4}. For example, phospholipid abnormalities are linked with pathological mechanisms of Alzheimer disease³. In Huntington's disease, interaction of huntingtin fragments, a protein which undergoes trinucleotide repeat expansion in the disease state⁴, with phospholipid membrane components is correlated with alterations in synaptic trafficking⁵.

Determining how lipids act to define region-specificity within tissues is extremely challenging given the immense diversity of lipids. Further, the specific function of any given lipid is likely influenced by cell type and cell microenvironment. Currently, little is known about the distribution of lipid classes (phospholipids, sphingolipids, and glycerolipids), even in the simplest division of brain regions, such as the cerebellum, hippocampus, striatum, and cortex⁶. Within these regions, functional disparities may be due to the heterogeneous distribution of cells or the variable interactions amongst the cell types. The variety of cellular distributions and interactions is clear when brain tissue is visualized with simple chemical and cell-specific immunohistochemical stains. However, these differences are difficult to categorize because cell-cell interactions exist both within and between brain regions. Thus, regional differences must be examined within the context of the entire tissue.

Mass spectrometry imaging (MSI) has emerged as a technique that is well suited to better understand lipid distribution within the brain⁷. Previous reports have utilized Matrix Assisted Laser Desorption Ionization (MALDI) in which a matrix-treated tissue section is analyzed by rastering a laser over the surface to generate mass spectra for each x-y position⁸. MSI directly interrogates the lipid distributions within the tissues. MSI analysis maintains spatial information that is lost during homogenization and extraction used for chromatography linked methods (e.g. LC/MS, CE/MS, or GC/MS)⁹ because these methods require massing multiple cell-types and regions to gain enough sample mass. Several studies have shown that MALDI-MSI can be used to directly visualize the localization of endogenous metabolites^{10,11} and proteins¹². This method is of particular usefulness for glycerol phospholipids^{13,14,15} in cells and tissues. Previous reports^{16,17,18} suggest that MSI has the potential to resolve cell type enrichment in the brain and that individual ions cannot resolve specific cell types or regions within the complex structure of brain tissue. Recently, Sugiura *et al*¹⁶ reported the visualization and localization of individual lipid species in neural cells, including several polyunsaturated fatty acids-glycerophospholipids (PUFA-GPLs), in different brain regions using single ion mapping through the optical observation of successive hematoxylin-eosin (HE)-stained brain sections, suggesting that PC (diacyl-18:0/22:6) was selectively detected in Purkinje cells and in molecular layers in which dendrites of Purkinje cells exist but did not resolve glial cells.

Here, we utilized a matrix-free surface mass spectrometry approach, nanostructure initiator mass spectrometry (NIMS)^{19,20,21}, to determine if multiple lipid ions and multivariate statistical methods can be used to generate a 'fingerprint' to discriminate between neuronal- and glial-enriched brain regions within a brain section. Sequential sections of tissues are used staining tissue to identify cell type enrichment and for NIMS imaging to define patterns of phospholipid intensity.

MATERIALS AND METHODS

Specimen attainment and handling

All animal handling and protocols were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Lawrence Berkeley National Laboratories Animal Welfare and Research Committee (IUCAC/AWAR Protocol # 27404).

Male Balb/C mice between the ages of 12 and 18 weeks were sacrificed and their brains rapidly retrieved. Gross segmentation was accomplished by placing the brain in a chilled stainless steel, sagittal mouse brain matrix (Zivic; Pittsburgh, PA) with 0.5 mm slice intervals. To achieve a flat, representative brain section, a 2 mm segment was taken from the midline on either side of the brain using ultrathin razor blades, transferred gently to a plastic transfer spatula and placed immediately into a well of an aluminum block prechilled to -14°C ²². Each segment was surrounded by OCT (Tissue Tek; Torrance CA), covered with a mounting chuck, and allowed to solidify for approximately 10 minutes within the cryochamber. The specimen was then removed with the removal apparatus and surface defects in the OCT were corrected by spreading a thin layer of OCT over the brain surface with the plastic transfer spatula. Mounted brain was allowed to equilibrate to cutting temperature on the chuck mount for 15 to 30 minutes.

To attain 5 μm slices, the cryostat was set to -14°C chamber temperature, -10°C mount temperature, with a 3.3 vacuum setting (Leica 1950 Cryostat, Leica Instruments; Nussloch, Germany). A 70 μm curl-guard was adjusted as needed to achieve flat, non-deformed sections. Flat sections were transferred to either Histoprep charged mounting slides (VWR International) or silicon NIMS chips²³. At least 7 glass Histoprep mounting slides with 2 brain sections per slide were collected on either side of the NIMS chip. At least 2 sections were transferred to the NIMS chip per brain, often 3 or more for use in calibration of the instrument. This collection method allowed for staining of approximately 100 μm of tissue on either side of the NIMS chip section.

Chemical Tissue Staining and Immunohistochemistry

Sections were dried on Histoprep glass slides overnight at room temperature or for a minimum of 3 hours at 37°C before further use. Tissue was rehydrated in PBS for 5 minutes at room temperature and blocked in PBS containing 0.1% Triton-X 100 (PBSTx) and 5% Horse Serum for 1 hour at room temperature. Antibodies against glial projections (anti-Glial fibrillary acidic protein, anti-GFAP-Cy3), neuron nuclei (anti-neuronal nuclei, anti-NeuN-Alexa 488) or neuronal projections (anti-microtubule-associated protein 2, anti-MAP2) were used to visualize brain regions. For 4-color stains containing MAP2, NeuN, GFAP and DAPI (a fluorescent chemical stain), sections were incubated with anti-MAP2 (diluted 1:200 in PBSTx) for 30 minutes, washed 3 times in PBSTx for 10 minutes each, and incubated with goat anti-mouse-Alexa 633 secondary for 1 hour at room temperature. Secondary antibody was washed extensively overnight in PBSTx and the tissue was stained with GFAP (1:400 in PBSTx) and NeuN (1:300 in PBSTx) for 30 minutes. After washing (3 times for 5 minutes in PBS), slides were mounted in Vectashield containing DAPI (Vector; Burlingame, CA) and imaged on a Zeiss 710 confocal microscope (Carl Zeiss; Gottingen, Germany). For whole brain images, z-stacked, tiled images at 20x objective were obtained and a maximum intensity projection of the z-stacks was isolated to adjust for focal plane shift over the sample. Immunohistology staining in which only a 3-color staining was employed eliminated the anti-MAP2 and secondary antibodies; the remaining protocol was the same.

Cresyl violet was used to quickly stain the brains to show gross morphology. Cresyl violet was diluted to 1% w/v in water containing 1% glacial acidic acid. For staining, a dried slide was placed in a 50 mL falcon tube containing 20 mL cresyl violet solution. The tube was inverted 3 times and the slide transferred to a primary wash (20 mL water in a 50 mL falcon tube) followed by a secondary wash (30 mL water in a 50 mL falcon tube). Washes were inverted 3 – 5 times until excess stain was fully removed. To destain, slides were transferred to a 50 mL falcon tube containing 70% ethanol, inverted twice and then transferred to a 50 mL falcon tube containing 100% ethanol (optional). Slides were allowed to dry and imaged on an AxioCam MRc5 5 megapixel camera (Carl Zeiss; Gottingen, Germany) with Olympus SZ61 dissection microscope (Olympus America; Center Valley, PA).

NIMS surface preparation

The preparation of nanostructure-initiator mass spectrometry (NIMS) surfaces has been described in detail elsewhere²³. Briefly, 4" silicon wafers (single-sided polished P/Boron, orientation <1-0-0>, resistivity 0.01–0.02 Ω cm, thickness 525 ± 25 μ m) were obtained from Silicon Quest International (Santa Clara, CA). The silicon wafers were cut into 70×70 mm square, and washed with 100% methanol solution for etching and coating steps. The etching process was done with 25% hydrofluoric acid in ethanol by using a customized Teflon etching chamber under constant current of 2.4 A for 15 min. Etched chips were subjected to coating procedure with 200 μ l of initiator liquid (bis(heptadecafluoro-1,1,2,2-tetrahydrodecyl) tetramethyl-disiloxane) purchased from Gelest (Morrisville, PA).

Imaging mass spectrometry

Nanostructure-initiator mass spectrometry (NIMS) imaging was performed on a 5800 TOF/TOF mass analyzer system (AB Sciex; Foster City, CA) in positive reflector mode. The third harmonic generation of a Nd:YAG laser (355 nm) was applied at a repetition rate of 200 Hz with ~20 shots per spot. A full mass spectrum ranging from 50 to 2000 m/z was acquisitioned for every pixel at an x-y step-size of 25–50 μ m. Four spots, located in the extreme corners of the chip, were chosen for calibration with peptide mass standards kit (AnaSpec; San Jose, CA) before the brains were imaged. Imaging data was stored in the Analyze 7.5 data format (Mayo Foundation; Rochester, MN).

2D image construction and statistical analysis

All following steps were executed for integrating multiple pixels into 2D images using custom-made scripts in the MATLAB (The MathWorks; Natick, MA) programming language. First, the Analyze 7.5 image files were parsed and imported by using a custom script. The script read the spectra at each x-y coordinate of each position and stored the intensity information in a 2D sparse array. The first dimension represents all the pixels, and the second dimension represents all the m/z values. Following a peak smoothing process with a Gaussian point-spread function ($\delta = 0.2$ Da), the peaks of 700 m/z–900 m/z were selected for all representative phospholipid ions (PE, PC, and PS). All the m/z values in each pixel were aligned with bin size of ± 0.3 Da. Non-negative matrix factorization (NMF) was used for the multivariate analysis. First, using the multiplicative update algorithm, twenty replicates were performed from random starting values. The best of these parameters was then used as the initial guess where the alternating least squares algorithm was used. The non-negative factors, W and H, were used to represent regions of the data in a reduced dimension. W is a 397980 (x•y•z) by 30 matrix. H is a 200 by 30 matrix, where 30 is the number of dimensions specified in the factorized model and 200 is the number of ions chosen for analysis. W can be easily reshaped into a 134 (x) by 99 (y) by 30 (z) by 30 (factors) matrix for 3D visualization and analysis. H is the loadings matrix that shows the contribution of each of the 200 ions towards each of the 30 components.

Liquid chromatography-tandem mass spectrometry

Whole mouse brain was extracted with a pre-cooled methanol–isopropanol–water (3:3:2) mixture following lyophilization in a FreeZone 2.5 lyophilizer (Labconco, Kansas City, MO, USA) and homogenization using a Mini-Beadbeater (BioSpec Products, Bartlesville, OK, USA). Chemicals were purchased from Sigma-Aldrich or Honeywell (Morristown, NJ, USA) and were of the highest purity. Normal-phase LC-MS/MS were performed on a Acquity UPLC HILIC column (Waters Corporation, Milford, MA, USA) with 1.7 μ m particles at a flow rate of 40 μ l/min. Chromatography was performed as follows (buffer A: H₂O with 5 mM ammonium acetate; buffer B: 90% acetonitrile with 5 mM ammonium acetate): equilibration in 100% buffer B for 5 minutes, 100–45% buffer B gradient over 30

minutes and isocratic elution with 45% buffer B for 10 minutes. Data were collected using an Agilent ESI-QTOF and a capillary voltage of 4000 V. Fragmentations were acquired with 10, 20, 40 V collision energy.

RESULTS AND DISCUSSION

NIMS imaging of brain sections

NIMS was used to obtain spatially resolved lipid distributions (Fig. 1). Whole brains from BALB/c mice were segmented ± 2 mm from the midline, fast-frozen, and sliced in 5 μ m sagittal sections in which the vertical plane was parallel to the midline. One slice was reserved for the metabolic analysis by NIMS, while the adjacent slices were collected on glass slides for detailed histology. To obtain high quality NIMS images, the flash frozen 5 μ m slices were immediately transferred to a NIMS chip. Rastering of the laser across the brain slice surface vaporizes tissue at the tissue/initiator interface to generate gas phase ions (Fig. 1). Rich spectra obtained were adequately consistent with MALDI results^{15,16}. The majority of our analysis focused on K⁺ adducts of GPLs^{15,16} to avoid adduct redundancy.

Region specific structures in the brain are visualized using chemical stains and antibodies

To delineate brain regions for later comparison with NIMS images, histological stains were performed on brain slices immediately adjacent to those applied to the NIMS chip. The position and morphology of brain regions were grossly determined based on subtle differences in the arrangement and concentration of cresyl violet (also known as Nissl stain). Areas of high neuronal density were particularly prominent in the olfactory bulb, hippocampus, and cerebellum (yellow, dark blue and pink respectively in Fig. S1). Nissl lightly stained the glial-rich corpus callosum, which is located between the cortex, hippocampus, and striatum (Fig. S1-A, light blue and green, respectively), while the cortical and striatal structures were poorly defined. Thus, Nissl staining was highly effective in distinguishing cell patterning, as well as the cell density, cell type, and cell arrangement in specific brain regions²⁴. The region-specific structures highlighted by this stain correlated well with the Mouse Brain Atlas²⁵.

Specific antibody staining identified the cell microstructures in brain slices. GFAP-Cy3 (glial fibrillary acidic protein) stained the glial cells²⁶, which formed elaborate networks of projections throughout the brain (Fig. S1-C, red). The glial networks were most pronounced in the cerebellar fissures, hippocampus, corpus callosum, and the olfactory bulb. Neuronal bodies (Fig. S1-D, NeuN-Alexa 488 in green), and the corresponding neuronal projections (Fig. S1-E, MAP2K-Alexa 644 in purple) had distinct, region-specific patterns within the cerebellum, hippocampus and olfactory bulb. Overlay of these distinct microstructures highlighted the relationships among glial cells, neurons, and their connections (Fig. 2A).

NIMS imaging of a tissue section resolves of brain-region and glial enriched regions

Although MALDI-MSI ion abundances have been shown to directly correlate to the relative levels of PC molecular species within a microdissected region analyzed by LC/MS/MS, relative quantification using mass spectrometry remains a major challenge^{16, 27}. It is common to detect multiple ions from a given lipid and the distribution of the ions ($[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$) is likely influenced by the salt concentrations within the various regions. To minimize this matrix effect, we focused on K⁺ adducts of 18 lipid ions which were identified by tandem mass spectrometry and/or reported by references. This adduct choice reduced redundancy as K⁺ adducts were the dominant form in NIMS as reported in MALDI¹⁶.

Previous reports have demonstrated the differential expression of various GPLs using MALDI, providing regional expression of individual ions over mouse brain^{12,27, 28, 29, 30, 31}. Likewise, NIMS is capable of detecting many of the same ions with additional detectability of small ions³². Many of the same lipids reported by Sugiura *et al*¹⁶ are also detected using NIMS including many of the diacylphospholipids (Table 1) and are found specifically in either glial-rich and neuron-rich brain regions (Fig. 2). For example, the areas within the sections that are rich in glial cells, as defined by parallel immunohistology, had an intense signal for 778.6 *m/z*, while other ions were specific for neuron-rich brain regions (e.g. 868.6 *m/z*) or distributed ubiquitously within either cell type (e.g. 826.6 *m/z*) (Fig. 2B). The ion 778.6 *m/z*, which exhibits highest signal intensity in corpus callosum, can be assigned to protonated form of sulfatide with diacyl chain (34:1) and is associated with the myelin sheath in glial axonal projections³³. Ions 826.6 and 868.6 *m/z* are assigned as potassium adducts of phosphatidylcholines (diacyl-18:0/18:1 and diacyl-18:2/22:6 respectively)^{16,27}. The PC (diacyl-18:0/18:1), known to accumulate in white matter during myelin formation¹⁶, was ubiquitous throughout the brain section compared to the sulfatide (ST-34:1), which is specific to corpus callosum. In comparison, we noticed that docosahexaenoate (DHA)-containing PCs (diacyl-18:2/22:6) were located in the gray-matter of the cortex in neuronal-rich regions of the brain section, as defined by histochemical staining.

To determine if resolution of glial enriched brain regions of section could be improved using multiple ions, we mapped three ions that exhibited cell-type intensified localization as follows: glial cell-enriched regions (778.6 *m/z*), neuronal cell-enriched regions (868.6 *m/z*) or both regions (822.6 *m/z*). The combined mass spectra image (Fig. 2) moderately improved brain region identification. Regions that are associated specifically with high glial and neuronal cell density were fairly easily distinguished. In certain tissue regions, two or more ions co-localized, creating a superimposed region on the ion map. For example, in the glial cell-rich region of frontal cortex, a yellow pseudo-colored area, represents equal localization of 806.6 and 822.6 *m/z*. The ion 822.6 *m/z* was found unaccompanied in cerebellar arbor by either of the other two ions. Interestingly, the ion 868.6 *m/z* was ubiquitously present throughout the brain. However, this ion was found in significantly lower levels within the glia-rich regions, suggesting the ion may have higher expression in neuronal cells.

Given that additional ions would improve delineation of brain regions within the section, we focused on 18 abundant ions that could be annotated as monoisotopic potassium adducts (Table 1). However, one cannot directly visualize the relative contribution of 18 ions simultaneously. Thus, we applied multivariate statistical tools to capture representative patterns from metabolite “sets”, which collectively lead to more specific regional characteristics in different brain regions. Metabolite “sets” are defined as those metabolites that are frequently detected together within a tissue region within a certain intensity range.

NIMS imaging coupled to multivariate statistical analysis of larger numbers of lipids improves brain-region and cell-type specificity

TRANSITION involving “sets” to components - The mass spectrum of each pixel within the NIMS two dimensional dataset was split into 6 components by non-negative matrix factorization (NMF) based on the 18 annotated potassium adducts (Table 1 and Fig. 3A). This number of components sufficiently represented most peak patterns that were spatially variable (Fig. S2). Thus, few components are specific for a given region; however, overlap of multiple components reveals region-specific lipid patterns. For example, among the 6 components, 5 and 6 could be closely linked to corpus callosum and fimbria while components 1, 3 and 4 strongly localized to the external cerebellar fissures.

As an example, the reconstructed NMF image comprised of components 4, 5, and 6 (Fig. 3B, pseudo-colored red, green and blue respectively) presented detailed correlation of ions with specific cell types within brain regions. These patterns closely matched regions visualized by cresyl violet and specific immunohistology stains (Fig. 2 and Fig. S1). The striatal caudate putamen area (Fig. 3B) contains glial projections surrounding the fimbria (teal, an overlay of component 5 and 6) that infiltrate the caudate putamen. Similar ion correlations exist in the glial-rich corpus callosum but not in the glial projections within the hippocampus, or those in the cerebellar arbor (Fig. 3B) suggesting that glia exhibit differential lipid profiles based on brain region. Several neuron-rich areas of the brain section appear in a single color (red-component 4, Fig. 3B), implying these regions share lipid-profile similarity, which is substantially different from glial regions. NMF imaging of the hippocampus differentiates the SVG, an area rich in neuroprogenitor cells (red, component 4), from the surrounding glial-rich region (green, component 5). However, other neuronal-rich regions appear to contain multiple components, implicating differing lipid profiles in the specific function of these regions (Fig. S2).

Surprisingly, the NMF analysis revealed compositional differences between the frontal cortex (pseudo-colored green, consisting of component 5) and the dorsal cortex (pseudo-colored red, consisting of component 4) that are not detected using the single and multiple ions (Fig. 2) nor in the histochemical stains. While not typically differentiated using simple histochemical stains, these regions have important functional differences. In our NMF analysis, we clearly see a bifurcation between the somatomotor component, in the frontal cortex, and the retrosplenial area of the dorsal cortex²⁵. These regions have distinctly different functions; they are responsible for control and pacing voluntary movements³⁴ and cognition or emotional processing³⁵, respectively.

Similarly, the pons and medulla are marked by independent component 6 localization (blue) while the midbrain contains component 4 with minor expression of component 5; these regions stain similarly using the simple immunohistochemical stains for glia and neurons (GFAP and NeuN respectively, Fig. S1). Complimentary, but unique, information is supplied by utilizing additional component visualization (such as components 1, 4, and 6 as illustrated in Fig. S3). Taken as a set of 6 components, patterning can yield unique lipid information about cell-types and inter-regional variation of lipid intensity.

CONCLUSION

NIMS imaging coupled to analysis of multiple ions provides an approach to resolve glial and neuronal cell enriched brain regions within a brain section. The resolution is greatly enhanced through application of multivariate statistical analysis (Nonnegative Matrix Factorization) of the 18 dominant K⁺ phospholipids. Strikingly, this analysis resolves other brain regions within the section that are difficult to distinguish using conventional stains but have known physiological differences including the somatomotor and retrosplenial components of the frontal and dorsal cortex and the pons region. This work suggests that using this approach with inclusion of larger numbers of lipids on multiple animals has the potential to greatly improve our understanding of regional and cell type specific variation in the brain.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. NIMS (Nanostructure initiator mass spectrometry) imaging scheme. Laser rastering ionized metabolites from nanoinitiator-associated tissue at defined spatial points. Distinct m/z ions from each point can then be mapped to create ion images.

Fig. 2. Individual ions exhibit differential brain patterning in the brain section. Immunohistochemical stain of a brain section illustrating areas of concentrated glial projections (A), montages of individual Ions (m/z 778.6, 826.6, 868.6) (B) and composite overlay (C) of the three (green, red, and blue respectively) provides improved region resolution of glial enrichment in brain regions.

Fig. 3. NMF Imaging yields a visualization of 3 components (A), out of 6 available components (Fig. S2). When overlaid, components 4 (in red), 5 (in green), and 6 (in blue) yield a multiplicative image with region-specific component combinations (B).

Table 1

Identified lipid molecules that are considered for NMF multivariate statistics. The references indicate the identical pattern of neutral loss in positive and negative ionization ms/ms respectively. The fragment, 163.0 m/z is the head group in potassium adducts form¹⁵. The characteristic fragments (acyl chains) in negative ionization mode were validated against lipid maps (<http://www.lipidmaps.org/>).

Compounds	m/z (K+ adduct)	Characteristic fragments (m/z) (+)	m/z (acetate adduct)	Characteristic fragments (m/z) (-)
SM(diacyl-18:0/18:1)	769.6		789.6	168.1 ^(15,36) , 715.7 ^(15,36)
PC(diacyl-16:0/16:1)	770.6	163.0, 711.5, 549.6	790.6	253.3, 255.3
PC(diacyl-16:0/16:0)	772.6	163.0, 713.5 ⁽¹⁶⁾ , 551.6	792.6	255.3
PC (diacyl-16:0/18:1)	798.6	163.0, 739.5 ⁽¹⁶⁾ , 615.6 ⁽¹⁶⁾	818.6	255.3, 281.3
PC(diacyl-16:0/18:0)	800.6	163.0, 741.6	820.6	255.3, 283.3
PC(diacyl-16:0/ 20:4)	820.6	163.0, 761.5 ⁽¹⁶⁾ , 637.5 ⁽¹⁶⁾	840.6	255.3, 303.3
PC(diacyl-16:0/ 20:3)	822.6	163.00, 763.7	842.6	255.3, 305.3
PC(diacyl-18:1/18:1)	824.6	163.00, 765.7	844.6	281.3
PC(diacyl-18:0/18:1)	826.6	163.0, 767.6 ⁽¹⁶⁾ , 605.6 ⁽¹⁶⁾	846.6	281.3, 283.3
PC(diacyl-16:0/22:6)	844.6	163.0, 785.5 ⁽¹⁶⁾ , 661.5 ⁽¹⁶⁾ , 623.6 ⁽¹⁶⁾	864.6	255.3, 327.3
PC(diacyl-18:1/20:4)	846.6	163.0, 787.5 ⁽¹⁶⁾ , 663.5 ⁽¹⁶⁾ , 624.6 ⁽¹⁶⁾	866.6	281.3, 303.3
PC(diacyl-18:0/20:4)	848.6	163.0, 789.7 ⁽¹⁶⁾ , 665.7 ⁽¹⁶⁾	868.6	283.3, 303.3
PC†(diacyl-38:3) *	850.6	163.0, 791.7	870.6	
SM(diacyl-18:1/24:1) *	851.6		871.6	
PC(diacyl-18:0/20:1)	852.6	163.0	872.6	283.3
PC(diacyl-18:2/22:6)	868.6	163.0, 770.6, 711.6	888.6	279.3, 327.3
PC(diacyl-18:1/22:6)	870.6	163.0, 811.6 ⁽¹⁶⁾ , 687.5 ⁽¹⁶⁾	890.6	281.3, 327.3
PC(diacyl-18:0/22:6)	872.6	163.0, 813.6 ⁽¹⁶⁾ , 689.6 ⁽¹⁶⁾	892.6	283.3, 327.3

* indicates putative annotation.