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Selective or Class-wide Mass Fingerprinting of Phosphatidylcholines and Cerebrosides from Lipid Mixtures by MALDI Mass Spectrometry

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Matrix assisted laser desorption/ionization (MALDI) mass spectrometry (MS) is a very effective method for lipid mass fingerprinting. However, MALDI MS suffered from spectral complexities, differential ionization efficiencies, and poor reproducibility when analyzing complex lipid mixtures without prior separation steps. Here, we aimed to find optimal MALDI sample preparation methods which enable selective or class-wide mass fingerprinting of two totally different lipid classes. In order to achieve this, various matrices with additives were tested against the mixture of phosphatidylcholine (PC) and cerebrosides (Cers) which are abundant in animal brain tissues and also of great interests in disease biology. Our results showed that, from complex lipid mixtures, 2,4,6-trihydroxyacetophenone (THAP) with $NaNO_3$ was a useful MALDI matrix for the class-wide fingerprinting of PC and Cers. In contrast, THAP efficiently generated PC-focused profiles and graphene oxide (GO) with $NaNO_3$ provided Cer-only profiles with reduced spectral complexity.

Key Words : MALDI MS, Phosphatidylcholine, Cerebroside, Graphene oxide

Introduction

Matrix assisted laser desorption/ionization (MALDI) mass spectrometry (MS) has been widely used for analyses of various classes of lipids due to its softness in ionization and its procedural simplicity.[1] However, MALDI MS possesses several challenging issues when analyzing complex lipid mixtures such as total lipid extracts and animal tissue specimens. First, many lipids form multiple ion species in MALDI mass spectra. Most frequent forms of ions in the positive ion mode are $[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$. These multiple ion species not only reduce sensitivity but also lead to isobaric interferences. Second, easily ionized lipid species can suppress ion signals from other lipids. For example, phosphatidylcholines (PCs) which are very abundant in animal tissues often suppress other lipid signals since PCs bear a permanent positive charge in their head groups and thus are easily ionized. Third, some of MALDI matrices form inhomogeneous crystal on a target and lead to poor spot-to-spot reproducibility in signals. For example, 2,5-dihydroxybenzoic acid (DHB), the most commonly used MALDI matrix in lipid analysis, forms needle-like crystals at the boundary of the sample spot. MALDI MS imaging study of DHB sample spots showed that a lipid profile at the boundary of the sample spot was totally different from that at the center of the spot.[2]

In order to reduce spectral complexity, many modifications on MALDI sample preparation methods have been investigated. Most frequently attempted modification was employing salt additives in sample preparation. Salt additives enhanced specific cation-adduct ion signals while suppressed others. Several cations including Li^+, Na^+, K^+, Cs^+, NH_4^+, and Ag^+ were employed in various forms of salts.[3-5] For example, with the addition cesium ions, spectral complexity was reduced and sensitivity was enhanced in lipid mass profiles.[3] In addition to this, effects of their counter anions such as chlorides, acetates, nitrates, and citrates in MALDI MS of lipids were also investigated.[5,6] Recently, Griffiths and Bunch systematically explored this salt additive effect in MALDI MS of lipids with a DHB matrix.[7] They found that sodium and potassium nitrates improved sensitivity and lithium salts were useful for product ion generation in MS/MS spectra. They also found that high concentration of nitrate salts (~80 mM) could be added without reducing spectral quality while other types of salts was applicable only with low concentrations (< 20 mM). However, their investigations were only limited to abundant PCs.[7] Another way for reducing complexity was employing desalting chemicals in MALDI MS analyses. For example, sol-gel/crown ether hybrid material was used to reduce the sodium adduct ion signals of oligonucleotides.[8] Lastly, lipid analysis in the negative ion mode could reduce the spectral complexity since most species were detected in the form of $[M-H]^-$. However, detectable lipid classes are limited and sensitivity is usually poor in the negative ion mode.

In order to obtain reasonable mass profiles for lipids of which ion signals are easily suppressed by other lipid species in complex lipid mixtures, several MALDI matrix alternatives were explored. Phosphatidylethanolamines (PEs) were very hard to detect in presence of PCs with conventional MALDI matrices such as CHCA or DHB. However, PEs were easily detected from crude lipid extracts if *para*-nitroaniline (PNA) or 9-aminoacridine (9-AA) was used as a matrix.[3,9] In addition, inorganic matrices were also employed for this purpose. For example, colloidal graphite and gold nanoparticle matrices were shown to selectively enhance ion signals of cerebrosides (Cers), a group of glycosphingolipids, from brain tissues and lipid extracts.[2,10]

In present study, various MALDI MS conditions were tested to find the optimal MALDI sample preparation methods for the selective ionization of PCs or Cers and also for class-wide mass fingerprinting of PCs and Cers (*i.e.*, detection of both PCs and Cers with comparable signal intensities) from the complex lipid mixtures with reduced spectral complexity. In order to achieve this, several previously reported or novel sample preparation methods were tested against a model lipid mixture of a PC and Cers. Our results showed that, from the complex lipid mixture, 2,4,6-trihydroxyacetophenone (THAP) matrix with $NaNO_3$ efficiently generated the class-wide fingerprints of a PC and Cers. In order to obtain selective fingerprints of PC and Cers with reduced spectral complexity, THAP-only and graphene oxide (GO) with $NaNO_3$ were the choices of MALDI MS sample preparation conditions, respectively.

Experimental

Materials. Porcine brain cerebroside extract in chloroform solution was purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). 1,2-Dipalmitoyl-*rac*-glycero-3-phosphocholine (PC 32:0), trifluoroacetic acid (TFA, 99% purity), 18-crown-6, and all organic acid MALDI matrices including α-cyano-4-hydroxycinnamic acid (CHCA), DHB, and THAP were obtained from Sigma-Aldrich (St. Louise, MO, USA). Aqueous graphene oxide (GO) solution was obtained from UniNanoTech Co. (Yongin, Korea). All salt additives were purchased from Sigma-Aldrich or Ducksan Pure Chemicals (Seoul, Korea). All solvents from Fisher Scientific (Fairlawn, NJ, USA) were used.

Sample Preparation for MALDI MS Analysis. The lipid samples were prepared in chloroform with the concentration of 1.0 mg/mL. Organic MALDI matrix solutions (25 mg/mL) were prepared in 80% (v/v) MeOH and 0.1% (v/v) TFA with or without additives. However, GO solution (0.05 mg/mL) was prepared in water first and various additives were directly added with a desired concentration. In case one or more additives were incorporated in the matrix solutions, final concentrations of additives in the matrix solutions are as follows; 20 mM of salt additives ($NaNO_3$ and KNO_3) and 10 mM of 18-crown-6. The lipid samples (0.5 μL) were first spotted onto a 384-well MALDI target plate (ASTA Inc., Suwon, Korea) followed by 0.5 μL organic MALDI matrix solution or by 2.0 μL of GO matrix solution. After spotting, a MALDI target was allowed to dry in a moderate vacuum (~50 Torr) at room temperature. Each type of sample had 5 replicates on a MALDI target.

MALDI MS Analysis of Lipids. MALDI-TOF and TOF/TOF mass spectra were obtained using ABI 4800 Plus MALDI-TOF/TOF analyzer (Applied Biosystems, Foster City, CA) controlled by the 4000 Series Explorer™ software. The mass spectrometer was operated in the positive reflectron mode with a 20 kV acceleration voltage, and signals from 1200 laser shots were used to generate a spectrum from each spot. In case of MS/MS experiments, collision induced dissociation was performed using air as the collision gas at 1 kV of collision energy. Mass spectra obtained were further processed and analyzed using a DataExplorer V4.8 (Applied Biosystems) and a mMass software (http://www.mmass.org).[11]

Results and Discussion

MALDI Matrices and Additives. Among hundreds of MALDI organic matrices reported, DHB and THAP were selected for this study because DHB is the most commonly used and comprehensively studied matrix for lipid analyses and because THAP seems to generate ions over various classes of lipids and also showed good salt compatibility.[12] For selective mass fingerprinting of Cers, carbon-based nanomaterials were tested since Cers have shown to be selectively detected from the crude lipid mixture by using a colloidal graphite matrix.[2] Among recently developed carbon-based matrices, graphene oxide (GO)[13] and reduced graphene oxide (rGO)[14] were initially selected and tested. However, only GO was subjected to further optimization processes since preliminary results showed the better suitability of GO for lipid analyses.

Various salt additives which contained Li^+, Na^+, K^+, or Cs^+ as a cation, and acetate (OAc^-), chloride (Cl^-), or nitrate (NO_3^-) as a counter-anion were initially investigated. However, Li^+ and Cs^+-containing salts turned out to be inappropriate for our purpose because they could not suppress naturally occurring ions by MALDI ($[M+H]^+$, $[M+Na]^+$, and $[M+K]^+$) and thus increased spectral complexities. Among counter-anions tested, NO_3^--containing salts was the most effective additives in MALDI MS in terms of sensitivity and salt concentration tolerance as Griffiths and Bunch reported recently.[7] Therefore, $NaNO_3$ and KNO_3 were selected for further method optimization. In addition to salt additives, 18-crown-6 was also tested since 18-crown-6 has been used for adduct simplification in the analyses of bacteria species by MALDI MS.[15,16]

A Model Lipid Mixture. As a model system, a mixture of PC 32:0 and total Cer extracts (1:5 w/w, 1 mg/mL in total) was used. Reasons for employing a combination of single PC and multiple Cers were as follows. First, introduction of isobaric interferences caused by different classes of lipids was not desirable in this study since this could lead to misinterpretation on ionization efficiencies of lipids. Therefore, a single PC, PC 32:0 (MW 734.04) was incorporated into a model mixture, which didn't cause any isobaric interference with Cers. Second, in contrast, isobaric interference caused by different salt adduct ions was desirable since reduction in spectral complexity by salt additives needed to be monitored in this study. For this purpose, we included total brain Cer extracts in a model mixture, which effectively generated isobaric interferences between [α-Hydroxyl Cer $+Na]^+$ and [Non-hydroxyl Cer$+K]^+$ in certain MALDI sample preparation methods. Since single PC and more than 20 different Cers are present in a model mixture, mixing ratio was adjusted to 1:5 (w/w, PC: Cers).

Class-Wide Fingerprinting of a PC and Cers. In order to obtain mass fingerprints from both PC 32:0 and Cers,

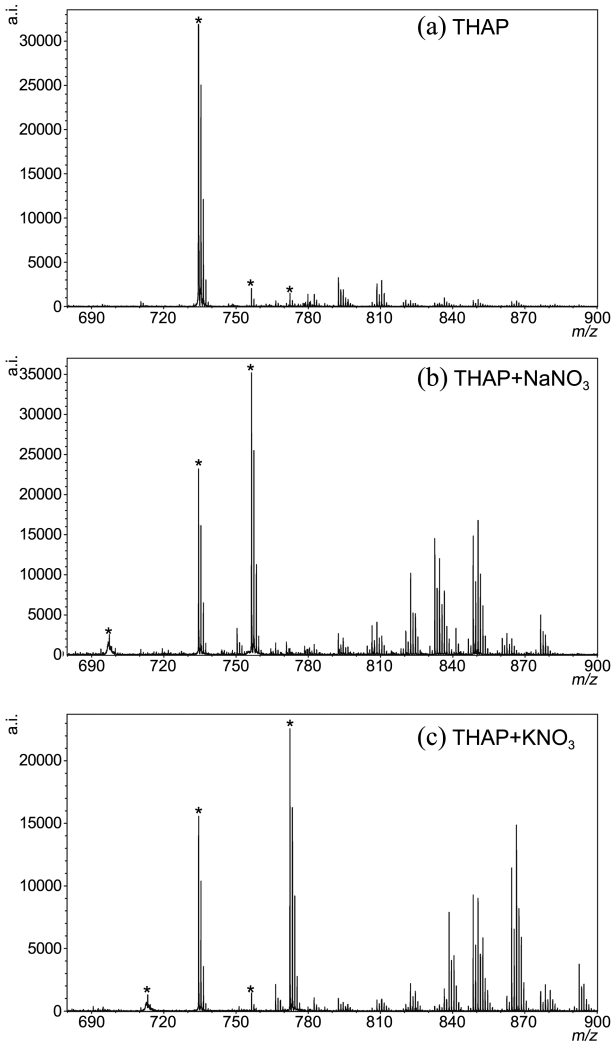

Figure 1. MALDI TOF mass spectra of a model lipid mixture (PC: Cers, 1:5 w/w) with (a) THAP, (b) THAP with 20 mM $NaNO_3$, and (c) THAP with 20 mM KNO_3. Mass spectral peaks from PC 32:0 were marked with asterisks (*). All matrix solutions in this figure contained 0.1% TFA (v/v).

Table 1. Mass peak assignments for major ions of a PC 32:0 and cerebrosides (Cer) in MALDI mass spectra of a model lipid mixture

m/z [a]	Possible Ions Assigned[b]
697	$[PC\ 32:0 - N(CH_3)_3 + Na]^+$
713	$[PC\ 32:0 - N(CH_3)_3 + K]^+$
734	$[PC\ 32:0 + H]^+$
750	$[Cer\ d18:1/18:0^b + Na]^+$
756	$[PC\ 32:0 + Na]^+$
766	$[Cer\ d18:1/18:0h^c + Na]^+$, $[Cer\ d18:1/18:0 + K]^+$
772	$[PC\ 32:0 + K]^+$
804	$[Cer\ d18:1/22:1 + Na]^+$
806	$[Cer\ d18:1/22:0 + Na]^+$
808	$[Cer\ d18:0/22:0 + Na]^+$
820	$[Cer\ d18:1/23:0 + Na]^+$, $[Cer\ d18:1/22:1 + K]^+$
822	$[Cer\ d18:1/22:0h + Na]^+$, $[Cer\ d18:1/22:0 + K]^+$
824	$[Cer\ d18:0/22:0h + Na]^+$, $[Cer\ d18:0/22:0 + K]^+$
832	$[Cer\ d18:1/24:1 + Na]^+$
836	$[Cer\ d18:1/23:0h + Na]^+$, $[Cer\ d18:1/23:0 + K]^+$
838	$[Cer\ d18:0/23:0h + Na]^+$, $[Cer\ d18:1/22:0h + K]^+$
848	$[Cer\ d18:1/24:1h + Na]^+$, $[Cer\ d18:1/25:0 + Na]^+$, $[Cer\ d18:1/24:1 + K]^+$
850	$[Cer\ d18:1/24:0h + Na]^+$
852	$[Cer\ d18:0/24:0h + Na]^+$, $[Cer\ d18:1/23:0h + Na]^+$
860	$[Cer\ d18:1/26:1 + Na]^+$
862	$[Cer\ d18:1/25:1h + Na]^+$
864	$[Cer\ d18:1/25:0h + Na]^+$, $[Cer\ d18:1/24:1h + K]^+$, $[Cer\ d18:1/25:0 + K]^+$
866	$[Cer\ d18:0/25:0h + Na]^+$, $[Cer\ d18:1/24:0h + K]^+$
876	$[Cer\ d18:1/26:1h + Na]^+$, $[Cer\ d18:1/26:1 + K]^+$
878	$[Cer\ d18:1/26:0h + Na]^+$, $[Cer\ d18:1/25:1h + K]^+$
880	$[Cer\ d18:0/26:0h + Na]^+$, $[Cer\ d18:1/25:0h + K]^+$

[a]Indicated *m/z*s are nominal *m/z*s. [b]Peak assignments were based on previously reported identifications[2] and MALDI MS/MS experiments. [c]Cer A/B corresponds to galactosylceramide sphingoid long chain base (A)/amide-linked fatty acid (B). [d]h corresponds to the hydroxyl group at the C2 position of the amide-linked fatty acid.

effects of salt additives were investigated since Cers have shown to undergo ionization through non-covalent cationization. Figure 1 shows MALDI mass spectra of a model lipid mixture with a THAP matrix, and Table 1 lists possible ion assignments for major peaks found in the mass spectra. Without any salt additive (Figure 1(a)), peaks from PC 32:0 were dominant in the mass spectrum, especially $[PC\ 32:0 + H]^+$ at *m/z* 734, and ions from Cers, which mainly appeared at *m/z* 800-900, are significantly suppressed. In contrast, marked signal enhancement for salt adduct ions of Cers was observed with the addition of $NaNO_3$ or KNO_3, and mass shifts due to sodium or potassium adduct ion formation were also obvious (Figure 1(b) and (c)). However, it should be also noted that added salts induced the cleavage of the PC head group which could increase the spectral complexity for the PC (peaks at *m/z* 697 and 713). For a 1.0 mg/mL of total lipid content (or with 0.5 μg of lipid spotted), 20 mM of sodium or potassium nitrate was found to be optimal in terms of sensitivity and signal reproducibility. Both $NaNO_3$ and KNO_3 produced Cer ions with similar intensities in the MS spectra. In the MS/MS spectra, however, almost no fragment ion except potassium ion was observed from a KNO_3-added sample while Cer-specific, sodiated fragment ions were clearly observed in a $NaNO_3$-added sample (Figure 2). Therefore, $NaNO_3$ seemed to have an advantage over KNO_3 in further structural elucidation by tandem MS.

DHB matrix with a salt additive also showed similar signal enhancement for Cers (data not shown), but DHB was less favored because of its poor spot-to-spot reproducibility. In addition, even though DHB with a salt produced more homogeneous crystals than DHB-only, there was still significant spectral inhomogeneity depending on laser sampling locations in a single spot. Therefore, we concluded that THAP with 0.1% TFA and $NaNO_3$ is the most suitable method for class-wide fingerprinting of PCs and Cers by MALDI MS.

Selective Fingerprinting of PCs. PC ions are easily detected by conventional MALDI protocols. For example,

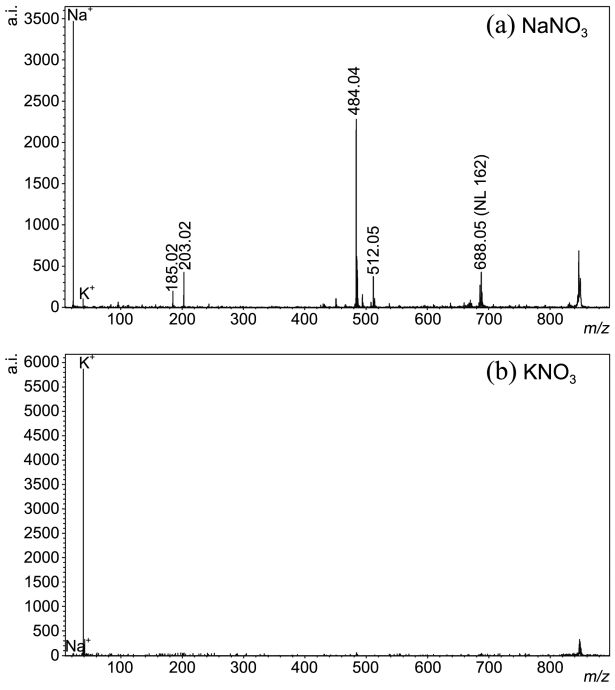

Figure 2. MALDI TOF/TOF mass spectra of ions detected at *m/z* 848 from MALDI mass spectra of a model lipid mixture with (a) THAP plus $NaNO_3$ matrix (Figure 1(b)) and (b) THAP plus KNO_3 matrix (Figure 1(c)). As listed in Table 1, the precursor ion at *m/z* 848 can be assigned as (a) $[Cer\ d18:1/24:1h + Na]^+$ or $[Cer\ d18:1/25:0 + Na]^+$ and (b) $[Cer\ d18:1/24:1 + K]^+$. In (a), the neutral loss (NL) of 162 corresponds to the loss of $C_6H_{10}O_5$ from the sphingosine long chain. Peaks at *m/z* 484 and at *m/z* 512 are the sodiated C_{18} sphingosine long chain and its aldehyde form. Mass peaks at *m/z* 185 and at *m/z* 203 correspond to $[C_6H_{10}O_5 + Na]^+$ and $[galactose + Na]^+$, respectively. All matrix solutions in this figure contained 0.1% TFA (v/v).

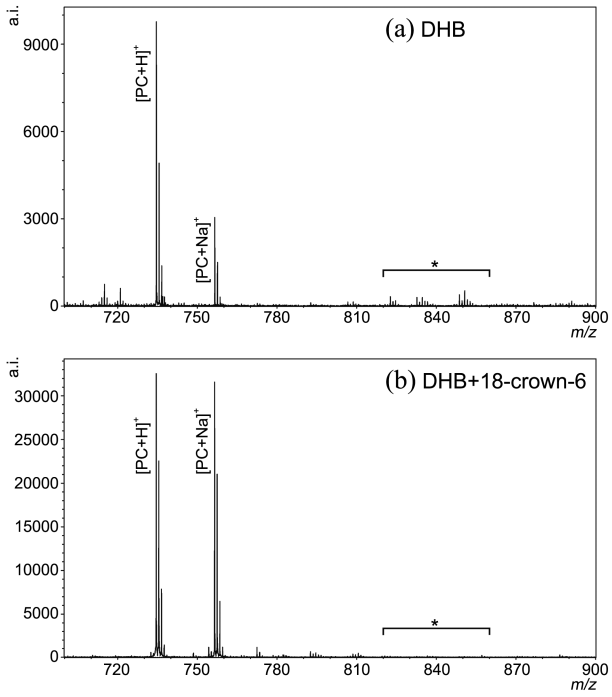

Figure 3. MALDI TOF mass spectra of a model lipid mixture (PC: Cers, 1:5 w/w) with (a) DHB and (b) DHB with 10 mM 18-crown-6. All matrix solutions in this figure contained 0.1% TFA (v/v).

PC 32:0 was selectively detected by a THAP matrix although very low intense signals of Cers were still present in the spectrum as shown in Figure 1(a). In order to achieve complete suppression of signals from Cers, a desalting chemical, 10 mM of 18-crown-6 was employed to the matrix solutions. However, 18-crown-6 didn't show a desalting effect for PCs with a THAP matrix and the mass spectrum obtained was very similar to Figure 1(a). In contrast, a combination of DHB and 18-crown-6 showed an interesting phenomenon. When 18-crown-6 was added to DHB, salt adduct ions of Cers were efficiently suppressed (Figure 3, the ranges marked with asterisks), but signals corresponding to $[PC\ 32:0 + Na]^+$ was significantly enhanced (Figure 3(b)). This trend was consistently observed through five replicate sample spots. In addition, shot-to-shot reproducibility was enhanced with the addition of 18-crown-6 since DHB crystals were formed more homogeneously. This differential suppression and enhancement may come from different adduct ion formation mechanisms between PCs and Cers. In case of zwitterionic PCs, sodium ions neutralize phosphate anions through an ion-ion interaction and this result in positive ions of PCs. In contrast, in case of Cers, sodium adduct ions are formed through a non-covalent interaction between sodium ions and functional groups of molecules. Galactose groups in Cers could be one of the major parts which interact with sodium ions. Therefore, this phenomenon suggests that 18-crown-6 could suppress non-covalent cationization in the gas phase but could not inhibit ionic cationization in MALDI processes. This finding needs to be confirmed by further study. Overall, both THAP-only and DHB with 18-crown-6 selectively ionized a PC from a mixture. However, a THAP-only method was chosen for the selective fingerprinting of PCs because DHB with 18-crown-6 enhanced salt adduct ions and thus could cause the increase in spectral complexity.

Selective Mass Fingerprinting of Cers. For selective ionization of Cers from a lipid mixture, an aqueous GO matrix solution was tested. Aqueous GO was used without TFA because TFA caused aggregation of GO particles. Similar to other carbon-based matrices, both GO and GO with a salt additive selectively generated Cer ions, ranging from $[Cer\ d18:1/18:0 + Na]^+$ at *m/z* 750 to $[Cer\ d18:0/26:0h + Na]^+$ or $[Cer\ d18:1/25:0h + K]^+$ at *m/z* 880, while no PC 32:0-originated ion was found in the mass spectrum (Figure 4(a)).

In mammalian lipid extracts, both non-hydroxyl and α-hydroxyl Cers are present although hydroxyl forms are more abundant than non-hydroxyl ones.[17] If MALDI of lipid mixtures generates both sodium and potassium adduct ions, hydroxyl and non-hydroxyl forms are hard to be distinguished in the mass spectra since the mass difference between non-hydroxyl and α-hydroxyl Cers are almost same as that between sodium and potassium. Therefore, reduction in the spectral complexity is particularly important in profiling Cers by MS. Figure 4(b), (c), and (d) are the magnified mass spectra to show effects of salt additives in detail. Reduction in spectral complexity by salt additives was obvious (Figure

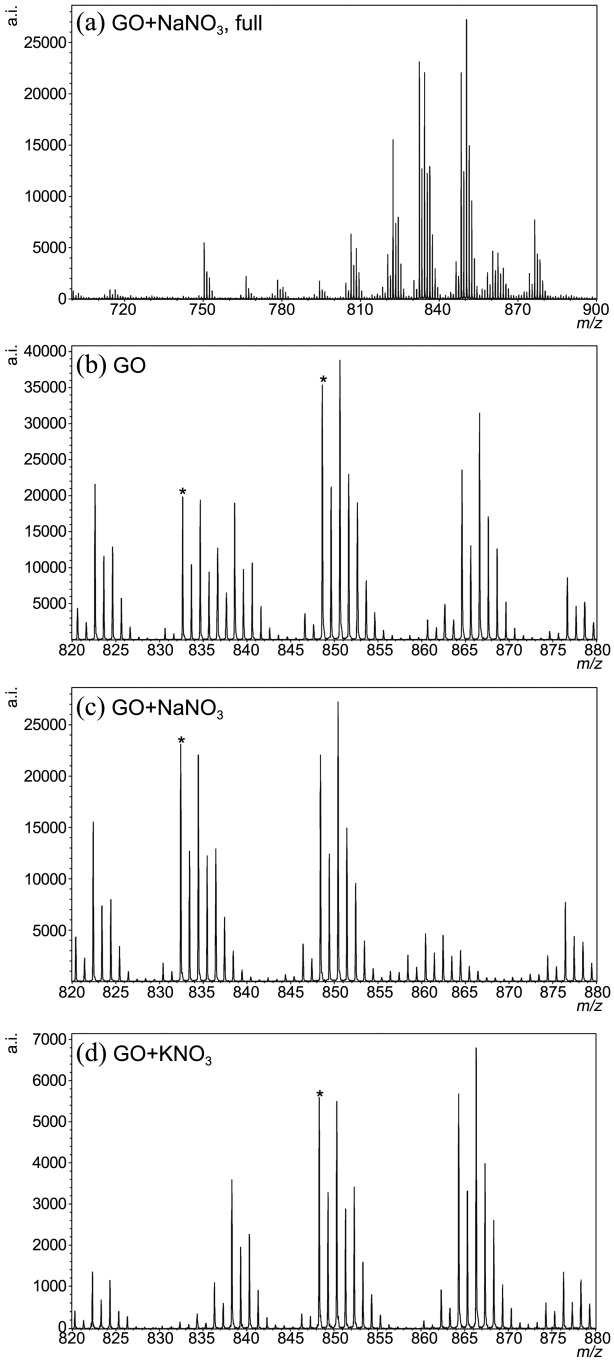

Figure 4. MALDI TOF mass spectra of a model lipid mixture (PC:Cers, 1:5 w/w) with (a), (c) GO with 20 mM $NaNO_3$, (b) GO only, and (d) GO with 20 mM KNO_3. (a) is a full spectrum covering the mass range where PC 32:0 and Cers could be detected. (b), (c), and (d) are magnified views of the mass range where major Cer ion species were detected. In (b), (c), and (d), mass spectral peaks which can be assigned as $[Cer\ d18:1/24:1 + Na]^+$ or $[Cer\ d18:1/24:1 + K]^+$ were marked with asterisks (*).

4(b) *versus* 4 (c) and (d)), and clear differences due to cation species were observed between $NaNO_3$-added and KNO_3-added samples, especially mass regions of *m/z* 830-835 and 864-870 (Figure 4(c) *versus* 4(d)). This selective cationization of Cers was further confirmed by MS/MS experiments and resulting mass spectra had very similar patterns with ones presented in Figure 2. Therefore, $NaNO_3$ was favored over KNO_3. In addition, with a GO matrix, base peak intensities were generally much higher in the spectra of $NaNO_3$-added samples than in those of KNO_3-added samples. However, this difference was not observed when using organic MALDI matrices.

Conclusion

In present study, MALDI sample preparation methods were tested and optimized for selective and class-wide ionization of PCs and Cers from a lipid mixture. Overall, THAP generated both class-wide and PC-focused profiles with good spot-to-spot reproducibility and GO was the choice of the matrix for the selective ionization of Cers. In addition, $NaNO_3$ was the effective salt additive for ionization of Cers because this salt enhanced ion signals from Cers, simplified mass spectral profiles of Cers, and produced informative fragment ions in the MS/MS spectra. Lastly, an effect of 18-crown-6 in MALDI MS of lipids was also explored and interesting phenomenon on lipid cationization was observed. However, it needs more comprehensive mechanistic studies to be confirmed. Future study will include application of developed protocols in this study to the various crude lipid extracts and similar optimization approaches to other lipid classes which are biologically important but hard to detect from complex lipid mixture, such as PEs and triacylglycerols.

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