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Technical Note

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Ammonium Sulfate Improves Detection of Hydrophilic Quaternary Ammonium Compounds through Decreased Ion Suppression in Matrix-Assisted Laser Desorption/Ionization Imaging Mass Spectrometry

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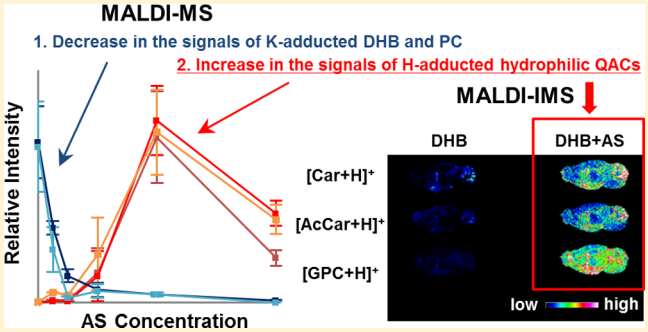
Supporting Information

ABSTRACT: Hydrophilic quaternary ammonium compounds (QACs) include derivatives of carnitine (Car) or choline, which are known to have essential bioactivities. Here we developed a technique for improving the detection of hydrophilic QACs using ammonium sulfate (AS) in matrix-assisted laser desorption/ionization-imaging mass spectrometry (MALDI-IMS). In MALDI mass spectrometry for brain homogenates, the addition of AS greatly increased the signal intensities of Car, acetylcarnitine (AcCar), and glycerophosphocholine (GPC) by approximately 300-, 700-, and 2500-fold. The marked improvement required a higher AS concentration than that needed for suppressing the potassium adduction on phosphatidylcholine and 2,5-dihydroxybenzoic acid. Adding AS also increased the signal intensities of Car, AcCar, and GPC by approximately 10-, 20-, and 40-fold in MALDI-IMS. Consequently, the distributions of five hydrophilic QACs (Car, AcCar, GPC, choline, and phosphocholine) were simultaneously visualized by this technique. The distinct mechanism from other techniques such as improved matrix application, derivatization, or postionization suggests the great potential of AS addition to achieve higher sensitivity of MALDI-IMS for various analytes.



Hydrophilic quaternary ammonium compounds (QACs) include derivatives of carnitine (Car) or choline, which are known to have essential bioactivities for energy production, lipid metabolism, and neurotransmission.[1−5] The metabolisms of those molecules are highly controlled by several transporters, and thus the gene deletion of those transporters leads to lethal conditions such as systemic carnitine deficiency[3] or respiratory failure due to perturbations of cholinergic signaling.[6] Moreover, the metabolisms of these molecules are closely associated.[5,7] For instance, a recent study suggested that therapeutic hypothermia provides its effect through a metabolic change resulting in a decrease of acetylcholine and an increase of Car in the same brain area.[5] These insights invoke the need for simultaneous distribution analyses of these molecules to elucidate their significant changes in various biological conditions.

Several studies have visualized the distribution of individual hydrophilic QACs using matrix-assisted laser desorption/ionization imaging mass spectrometry (MALDI-IMS), which provides label-free simultaneous analyses of multiple molecular distributions on tissue.[8,9] However, the sensitivity of MALDI-IMS for hydrophilic QACs is still not satisfactory. For instance, the signal obtained from acetylcarnitine (AcCar) in the brain is quite low and the signal-to-noise ratio (S/N) reported by Pirman et al. is not adequate for analyzing its metabolic change.[10,11] Moreover, further high sensitivity will be needed for the detection of small amounts of analytes at high spatial resolution (<1.0 μm).[12] Techniques that improve sensitivity for hydrophilic QACs are thus desired.

It is especially difficult to improve the signal of hydrophilic molecules in MALDI-IMS because the distribution of such molecules is easily affected by aqueous solution.[13,14] At present, derivatization and a matrix additive are used for several hydrophilic molecules.[9,15−17] Techniques for derivatization reported by Toue et al.,[17] Shariatgorji et al.,[9] and Manier et al.[15] succeeded only in several primary amines, and they require careful optimization in complicated protocols. The techniques also increase the risk to changing the spatial distribution and the chemical structure of analytes at each procedure. In contrast, matrix additives do not raise such a risk since the application on a sample can be performed simultaneously with that of the matrix.[16,18] It is thus necessary to find effective matrix additives in order to improve the sensitivity for hydrophilic QACs. Typical matrix additives such as chelating

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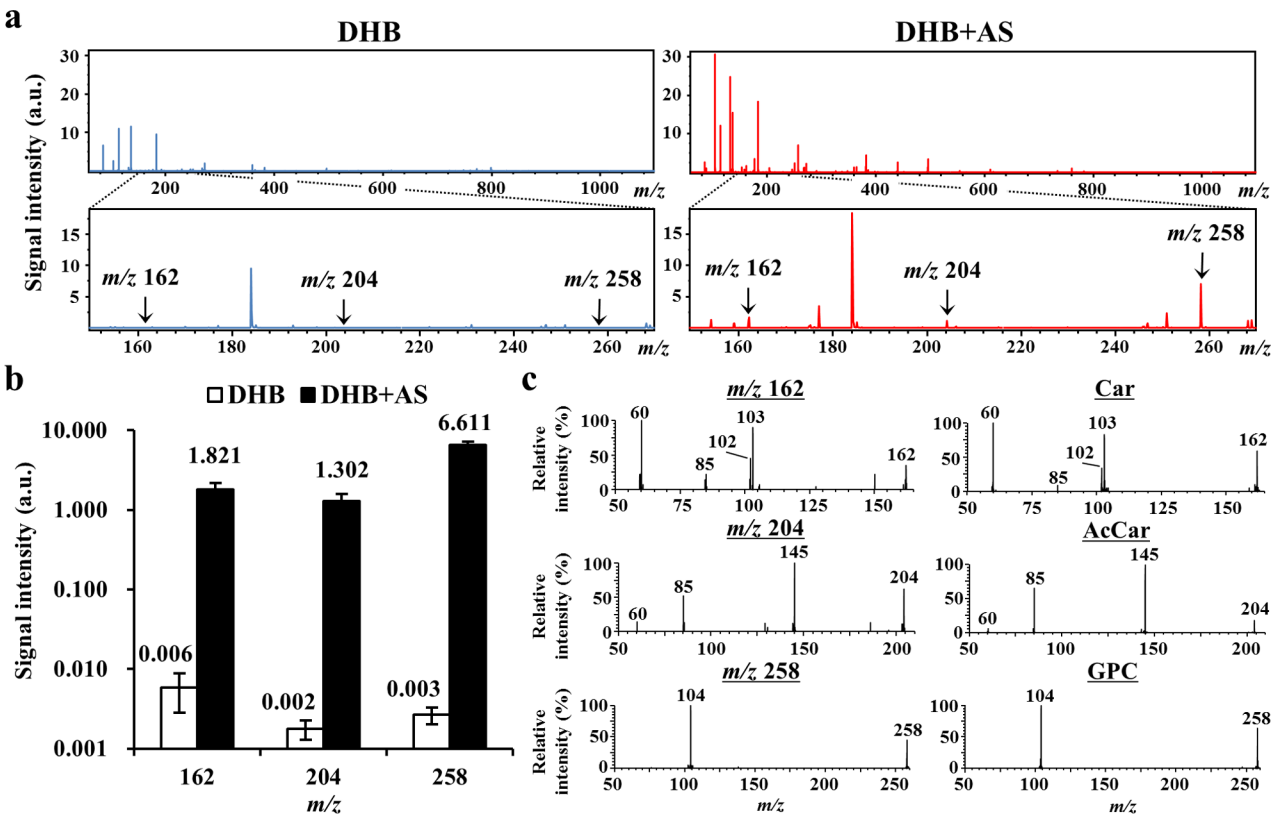

Figure 1. AS markedly increased the signal intensities of Car, AcCar, and GPC from mouse brain homogenates on MALDI-MS. (a) Representative averaged mass spectra obtained from mouse brain homogenate under the conditions using DHB only (left, blue) and DHB with 250 mM AS (right, red) in the entire measured range (m/z 60−1100, upper) and enlarged range at m/z 150−270 (lower). (b) The signal intensities of the peaks at m/z 162, 204, and 258 from mouse brain homogenates were compared with/without AS. Averaged intensities are shown above each bar. Error bar: standard deviation (SD; $n = 6$). (c) MS/MS spectra of m/z 162, 204, and 258 obtained from mouse brain homogenates (left) and standards (right).

agents and ammonium salts provides their effect through a reduction of matrix cluster ions by suppressing alkali metals in the sample.[16,19−21]

Ammonium sulfate (AS) is a unique matrix additive that improves the signal of various types of biomolecules including oligonucleotides,[22] peptides,[23,24] and glycolipids in MALDI mass spectrometry (MALDI-MS).[18] The improvement was mainly shown by Woods and her co-workers[18,23,25,26] and they also applied AS to lipid analysis in MALDI-IMS.[18,26] The improved detection provided by AS was suggested to be based on multiple effects including the prevention of interference from alkali metal ions,[18,26] and the mechanisms were not characterized as working specifically on particular molecules in the previous reports. We hypothesized that the signals derived from hydrophilic metabolites are also improved through the multiple effects of AS.

In the present study, we aimed to improve the sensitivity of MALDI-IMS for hydrophilic QACs. We used AS with 2,5-dihydroxybenzoic acid (DHB), which is capable of ionizing individual hydrophilic QACs,[5,10,11,27] to accomplish this, and by doing so we were able to perform a simultaneous distribution analysis of five hydrophilic QACs in mouse brain tissue.

EXPERIMENTAL SECTION

Animals. C57BL/6J male mice at 8 weeks old were purchased from Japan SLC (Hamamatsu, Japan). After cervical dislocation euthanasia, the brains of the mice were removed and immediately frozen in powder dry ice and stored at −80 °C. All animal experimental procedures were approved by the Experimental Animal Committee of the Hamamatsu University School of Medicine.

Tissue Preparation. Whole mouse brain was cut and homogenized in ultrapure water (500 mg tissue/mL) on ice. The homogenate was dispensed to 100 μL aliquots used for single measurement and stored at −80 °C until use. Brain homogenate was diluted to 100 mg tissue/mL by ultrapure water and mixed with an equal volume of DHB solution (50 mg/mL in 50% methanol containing five different concentrations of AS: 0, 16, 31, 63, 125, and 250 mM) before measurement. For sectioning, tissue was fixed on a stage using Optimal Cutting Temperature compound (Sakura Finetek Japan, Tokyo) and equilibrated at −20 °C in the cryostat (Leica CM1950, Leica Biosystems, Nussloch, Germany). Tissues were sectioned at 10 μm thick and thaw-mounted on conductive indium-tin-oxide (ITO)-coated glass slides at 100 Ω (Matsunami Glass Industries, Osaka, Japan).

MALDI-MS and MALDI-IMS. Measurement was performed with a MALDI-time-of-flight (TOF)/TOF mass spectrometer (ultraflex II, Bruker Daltonics) equipped with a smart beam (a 355 nm Nd:YAG laser) in reflectron mode. For the evaluation of the effect of AS on MALDI-MS, 1.0 μL of each sample−matrix mixture was dropped on ITO glass using siliconized tips and dried under a vacuum for 5 min.

For MALDI-IMS, 1.0 mL of DHB solution (50 mg/mL in 50% methanol) with/without 250 mM AS was sprayed on sections with an air-brush (Procon Boy FWA platinum; Mr. Hobby, Tokyo). Data were acquired from m/z 60 to 1100 in the positive ion mode. Raster scanning was performed at 150

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μm pitch. At each position, the 25 μm diameter laser irradiated a point 10 times and the obtained spectra at 20 points were summed up. The repetition rate was set at 100 Hz, and the relative laser power was set at 20%. Ion images were reconstructed with normalization using the total ion current and analyzed using flexImaging 4.0 software (Bruker Daltonics).

We performed MALDI tandem mass spectrometry (MS/MS) using a Mass Microscope, a prototype of iMScope equipped with a 355 nm Nd:YAG laser (Shimadzu, Kyoto, Japan).[28] Homogenates (100 mg tissue/mL) and standard solutions (100 μM in 5% ethanol) of carnitine (Car), acetylcarnitine (AcCar), and glycerophosphocholine (GPC) were mixed with DHB solution (50 mg/mL in 50% methanol containing 250 mM AS) and analyzed. The sample voltage and detector voltage were set at 3.5 kV and 2.1 kV, respectively. The relative laser power was set to 47%. The laser irradiated each position 200 times with 25 μm diameter at 1000 Hz repetition rate. The collision energy was optimized to obtain an intense pattern of fragment ions for each compound. Data were analyzed by Imaging MS Solution (version 1.01.02, Shimadzu).

RESULTS AND DISCUSSION

The mass spectra obtained from the mouse brain homogenates were remarkably changed in the presence of 250 mM AS in the matrix solution (Figure 1a, upper). In particular, the signal intensities at m/z 162, 204, and 258 exhibited higher intensities (Figure 1a, right) by 300-, 700-, and 2500-fold and higher S/N by 200-, 700-, and 2900-fold compared to those obtained without AS (Figure 1b, Figure S-1). These signals were identified as $[Car + H]^+$, $[AcCar + H]^+$, and $[GPC + H]^+$ by MS/MS analysis (Figure 1c). AS also increased the signals derived from other hydrophilic QACs in the brain homogenates. The signals at m/z 104 and 184 correspond to $[choline + H]^+$ and $[phosphocholine + H]^+$, were increased by 17- and 2-fold and higher S/N by 12- and 2-fold, respectively, compared to those obtained without AS (Figure S-2). The signal at m/z 146, which corresponds to both $[acetylcholine + H]^+$ and $[\gamma\text{-butyrobetaine} + H]^+$, was increased by 35-fold and higher S/N by 37-fold compared to those obtained without AS (Figure S-2). It should be noted that acetycholine and γ-butyrobetaine are structural isomers (Figure S-3) and reported to exist at similar level in rat brain.[29−31]

AS greatly improved detection of Car, AcCar, and GPC in the mouse brain homogenates. These three molecules each have a common quaternary ammonium structure, which contains a stable positive charge (Figure S-3). The structure is known to effectively take net charges from a desorbed cluster produced in MALDI-MS.[32] These three compounds are thus considered to be markedly sensitive to the improved detection by AS. The increase in the signal intensities of the other hydrophilic QACs also supports our hypothesis.

Car, AcCar, and GPC in each standard solution at the estimated concentration of Car in the homogenate sample (5 μM)[5] were further examined, and they exhibited higher signal intensities by 7-, 2-, and 8-fold and higher S/N by 7-, 2-, and 7-fold by AS, respectively (Figure S-4). The increase ratios were much lower than those observed in homogenate samples. Therefore, AS has the mechanism that decreases ion suppression derived from biological components as well as other mechanisms that increase signals of standards. The result also suggests the major contribution of the decreased ion suppression to the improved detection of Car, AcCar, and GPC.

In order to evaluate the relationship between the AS concentration and the improvement of the three signals, we mixed the matrix solution containing various AS concentrations (0, 16, 31, 63, 125, and 250 mM) with mouse brain homogenate and analyzed the results. The visual aspects of the crystallization of droplets clearly varied depending on the AS concentration (Figure 2a). The signal intensities of $[Car + H]^+$, $[AcCar + H]^+$, $[GPC + H]^+$, $[choline + H]^+$, and m/z 146 were increased by AS in a dose-dependent manner, and the signals were maximized at 125 mM (Figure 2b, Figure S-5a,b). The increase ratios at 31 mM on $[Car + H]^+$, $[AcCar + H]^+$, and $[GPC + H]^+$ were less than 5.0%, 1.5%, and 0.3% relative to the maximum increase ratio (defined as 100% at 125 mM) compared to 0 mM (Figure 2b). In contrast, the signal derived from $[phosphocholine + H]^+$ was increased and reached its maximum at 31 mM (Figure S-5a).

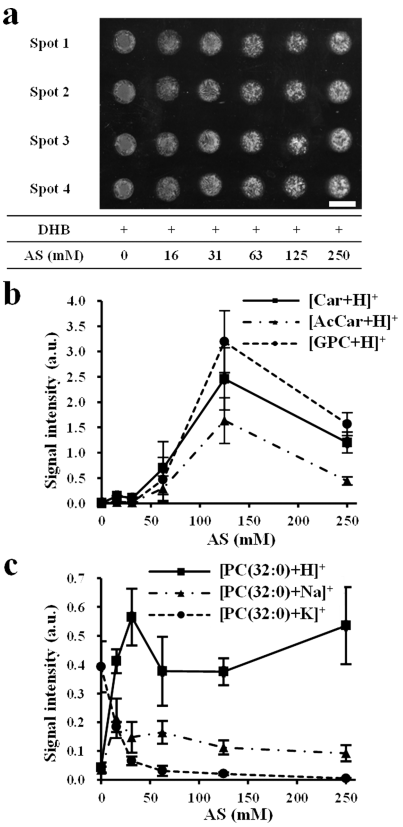

Figure 2. AS dose-dependently improves the signals of Car, AcCar, and GPC in mouse brain homogenates. (a) Optical image of all measured homogenate−matrix mixtures. Solute compositions of matrix solutions are shown under the image. Scale bar: 2 mm. (b) Intensity profiles of $[Car + H]^+$, $[AcCar + H]^+$, and $[GPC + H]^+$ depending on AS concentrations. (c) Intensity profiles of $[PC(32:0) + H]^+$, $[PC(32:0) + Na]^+$, and $[PC(32:0) + K]^+$ depending on AS concentrations. Error bar: SD; $n = 4$.

To characterize the mechanism of improvement on hydrophilic QACs, we also analyzed the signals derived from phosphatidylcholine (PC). AS promoted the formation of $[PC + H]^+$ instead of $[PC + K]^+$ as reported in a previous study (Figure S-6).[26] Among the signals derived from some PCs, we focused on PC(32:0), whose three adduct-form types are separate from any adduct ion forms of other PCs.[33] We

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analyzed the signal intensities with each adduct ion under the conditions with/without AS.

The signal derived from $[PC(32:0) + K]^+$ at m/z 773 continued to decrease and reached its minimum at 250 mM (Figure 2c). The suppression ratio was defined as 100% using the signal intensity at 250 mM compared to that at 0 mM, and the suppression ratio reached 84% relative to that obtained at 31 mM. On the other hand, the signal derived from $[PC(32:0) + H]^+$ detected at m/z 735 was increased by AS and reached its maximum at 31 mM (Figure 2c). The signal intensities of $[PC(32:0) + Na]^+$ at m/z 757 were decreased proportionally to the AS concentration, although the signal was increased in the presence of AS compared to that obtained without AS (Figure 2c). Compared to the intensity at 0 mM, sum of intensities for the three PC(32:0)-derived ions were increased at 16 and 31 mM while those in other concentrations did not show significant difference (Figure S-7).

We further analyzed the dose dependency of signals associated with DHB since the production of the matrix cluster with alkali metal ions was revealed to decrease the signals of analytes.[21,22] The signals at m/z 231, corresponding to $[DHB + 2K - H]^+$,[16] were greatly decreased by the addition of AS and hardly detected at higher than 31 mM. Compared to 0 mM, the suppression ratio at 31 mM reached 97% relative to the maximum suppression ratio (defined as 100% at 250 mM) (Figure S-8a). In contrast, the signals at m/z 137, corresponding to $[DHB - H_2O + H]^+$,[13] were increased by 2-fold at 63 mM and then started decreasing at higher concentrations; no affect was exhibited at 250 mM (Figure S-8b).

From our results, the marked improvement for the detection of Car, AcCar, and GPC by AS was found to require a higher concentration than that for suppressing potassium adduction on PC and DHB. In the present study, AS mainly improved signals of the three molecules at 63 mM and more (Figure 2b), and suppressed potassium interference at below 31 mM (Figure 2c and Figure S-8a). The concentration needed for suppressing potassium adduction can be explained by the potassium concentration in the sample homogenates (100 mg/mL) estimated as 9.5 mM, based on the value 95 μmol/g tissue reported for rat brain tissue.[34] At 63 mM and more, the suppression ratios of potassium adduction were obtained below 16% (Figure 2c and Figure S-8a). We thus speculate that the improving effect of AS at higher concentrations was provided by other mechanisms that decrease ion suppression. AS is frequently used for salting-out, which is an effect that decreases solubility of various molecules in aqueous solution by high ionic strength.[35] In the present study, AS was applied and concentrated on the surface of the samples. The concentrating step leads to salting-out of various molecules and promotes extraction of the three molecules into cocrystals with DHB. Therefore, we considered the salting-out as an important mechanism for decreasing ion suppression although the effect of AS addition for each analyte depends on the combined matrix (Figure S-9).

AS is expected to be useful for the spray-coating of matrix on tissue sections, which is generally used for MALDI-IMS. On the basis of our finding that a concentration higher than 31 mM is needed for obtaining the marked improving effect for homogenates and the result of a comparison of AS concentrations for mouse brain coronal sections (Figure S-10), we next used a 250 mM AS condition for MALDI-IMS in this study. The condition for the spray-coating clearly improved the mass spectra while maintaining the mouse brain structure (Figure S-11), and the averaged signal intensities of $[Car + H]^+$, $[AcCar + H]^+$, $[GPC + H]^+$, $[choline + H]^+$, $[phosphocholine + H]^+$, and m/z 146 were increased by 11-, 17-, 38-, 3-, 13-, and 4-fold (12-, 18-, 40-, 3-, 6-, and 7-fold increase in S/N), respectively (Figure 3a, Figure S-11a, lower). Moreover, Car, AcCar, GPC, phosphocholine, and m/z 146 were clearly visualized with AS, whereas they were hardly observed without AS (Figure 3b, Figure S-11b).

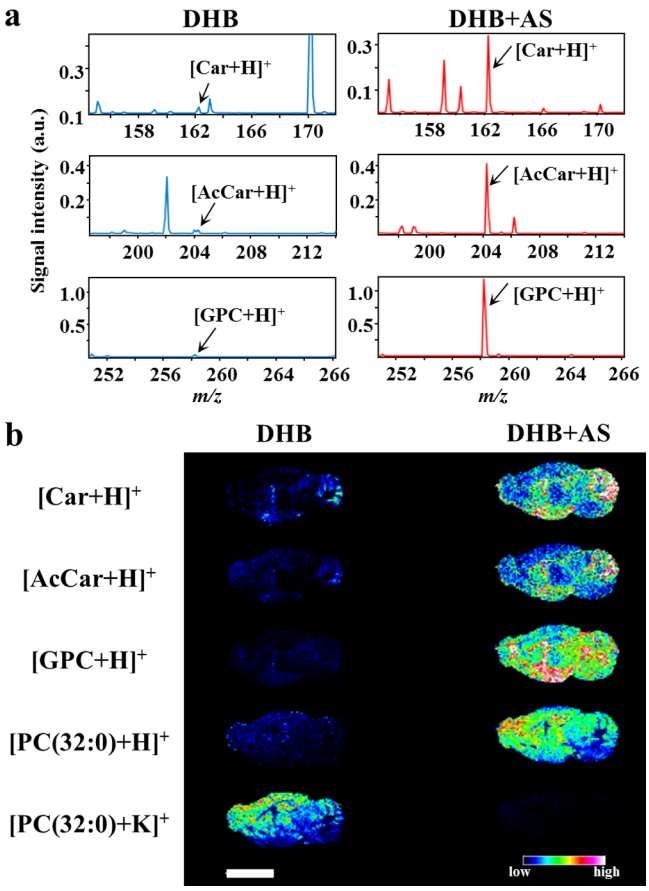

Figure 3. AS increased the signal intensities of Car, AcCar, and GPC from mouse brain sections; these molecules maintained their localization in MALDI-IMS. (a) Enlarged mass spectra obtained from mouse brain sections using DHB solution only (left, blue) and DHB solution containing 250 mM AS (right, red) for spray coating. Black arrows: peaks corresponding to $[Car + H]^+$ (top), $[AcCar + H]^+$ (center), and $[GPC + H]^+$ (bottom). (b) Ion images of $[Car + H]^+$, $[AcCar + H]^+$, $[GPC + H]^+$, $[PC(32:0) + H]^+$, and $[PC(32:0) + K]^+$ under the conditions with/without AS. Scale bar: 5 mm.

In contrast, the ion image of choline was almost the same between with and without AS (Figure S-11b). Car and AcCar were strongly detected in the cerebellum, striatum, and hypothalamus. GPC was strongly detected in the hypothalamus and granular cell layer of the olfactory bulb. PC(32:0) showed a similar distribution under both conditions, and major adduct ions were changed from potassium to proton (Figure 3b). Phosphocholine was detected throughout the entire region, and m/z 146 was strongly detected in the striatum, hippocampus, and cerebral cortex (Figure S-11b).

AS was shown to improve the sensitivity for hydrophilic QACs, especially Car, AcCar, and GPC, in MALDI-IMS. It is also apparent that AS provided its effects while maintaining the

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distributions of the QACs, since the present distributions of GPC and PC(32:0) are consistent with those reported in earlier studies.[9,33] Before the present study, the simultaneous analysis of five molecules including Car, AcCar, GPC, choline, and phosphocholine had not been achieved. The addition of AS to DHB is therefore a powerful tool for analyzing these molecules. The improved sensitivity allows a precise quantification and higher spatial resolution than conventional methods.

Since there are few currently available techniques for increasing signals without the migration of hydrophilic molecules, the technique using AS will be also a new way to improve the sensitivity of MALDI-IMS for these molecules. Here we explored the utility of AS for hydrophilic QACs in mouse brain, and further studies of other tissues or analytes are strongly expected. AS is capable of improving the detection of oligonucleotides,[22] peptides,[23,24] lipids,[18] and hydrophilic metabolites in MALDI. It can be used not only with DHB but also other matrices (e.g., 2,6-dihydroxyacetophenone[18,26] and α-cyano-4-hydroxycinnamic acid[24]). Moreover, recent novel techniques for obtaining high sensitivity (i.e., improved matrix application,[8,36,37] derivatization of analytes[9,15,17,38] and postionization using MALDI[39]) can be directly combined with the use of AS. Therefore, AS has great potential to achieve higher sensitivity of MALDI-IMS for various analytes.

CONCLUSION

The results of the present study revealed that AS improves the detection of hydrophilic QACs, especially Car, AcCar, and GPC, in mouse brain on MALDI. The improvement required higher concentrations than that for suppressing the interference of potassium. Successful visualization of hydrophilic QACs in tissue sections was also achieved through the improvement by AS.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b02672.

Supplementary materials and methods and data (PDF)

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Notes

The authors declare no competing financial interest.

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