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Keywords
MALDI, mass spectrometry imaging, matrix, metabolites, small molecules, 1, 5-diaminonaphthalene

Disciplines
Analytical Chemistry | Chemistry

Comments

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MALDI-MS analysis and imaging of small molecule metabolites with 1,5-diaminonaphthalene (DAN)

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1,5-diaminonaphthalene (DAN) has previously been reported as an effective matrix for matrix-assisted laser desorption ionization-mass spectrometry of phospholipids. In the current work, we investigate the use of DAN as a matrix for small metabolite analysis in negative ion mode. DAN was found to provide superior ionization to the compared matrices for MW $< \sim 400$ Da; however, 9-aminoacridine (9-AA) was found to be superior for a uridine diphosphate standard (MW 566 Da). DAN was also found to provide a more representative profile of a natural phospholipid mixture than 9-AA. Finally, DAN and 9-AA were applied for imaging of metabolites directly from corn leaf sections. Published 2014. This article is a U.S. Government work and is in the public domain in the USA.

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**Keywords:** MALDI; mass spectrometry imaging; matrix; metabolites; small molecules; 1,5-diaminonaphthalene

**Introduction**

The choice of matrix is critically important in matrix-assisted laser desorption ionization-mass spectrometry (MALDI-MS). Recent interest in metabolomic applications of MALDI-MS has resulted in the development of novel matrices and nanostructured substrate surfaces for the analysis of small molecules (MW $< \sim 1,000$ Da). Substrate surfaces based on nanoporous silicon\cite{1} and nanopost arrays\cite{2} have shown superior sensitivity for small molecule analyses; however, their imaging application is limited to very thin tissue samples ($< 10 \mu$m). Titanium dioxide nanoparticles\cite{3} and graphene oxide\cite{4} are emerging new matrices and have been successfully demonstrated for small molecule analysis. With regard to organic matrices, 9-aminoacridine (9-AA),\cite{5} 1,8-bis(dimethylamino)naphthalene (DMAN),\cite{6} and N-(1-naphthyl) ethylenediamine dinitrate\cite{7} have been reported to be useful for small molecule analysis, particularly for negative ion mode. Among these, the strong basicity of DMAN is suggested to be useful for MALDI-MS analysis of weakly acidic biomolecules; however, the vacuum instability of DMAN has become known to be a serious limitation, particularly for imaging applications.\cite{8}

Another basic matrix, 1,5-diaminonaphthalene (DAN), is known for in-source decay top-down sequencing of proteins and peptides in positive ion mode because of its hydrogen radical transfer capability.\cite{9,10} It has also been used for in-source fragmentation of oligonucleotides in negative ion mode.\cite{11} Recently, Thomas and coworkers reported that DAN provides superior ion signals for a wide range of lipids, in both positive and negative modes.\cite{8} However, there has been no report yet on the use of DAN for low-molecular weight (LMW) analytes (MW $< 600$ Da). The development of additional matrices for this mass range is highly desirable for the study of plant metabolic biology, especially for negative ions. Many metabolites within this mass range readily yield negative ions (e.g., primary metabolite small organic acids, phosphate sugars and nucleoside phosphates, among others). In the current study, we investigate the use of DAN for small molecule analysis in negative ion mode and compare its performance to other matrices.

A few LMW compounds representing several plant metabolite classes were used as test analytes in the current study: malic acid (MW 134), glutamic acid (MW 147), phosphoenolpyruvic acid (MW 168), ascorbic acid (MW 176) and uridine diphosphate glucose (MW 566). In addition to DAN, 9-AA, $\alpha$-cyanohydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB) were compared in the same experimental conditions. DAN and 9-AA were also compared for the negative mode analysis of an extract of soy phospholipids to evaluate their effectiveness in the analysis of medium molecular weight compounds (MW between 600 and $\sim 1000$ Da). Finally, DAN and 9-AA were applied for the chemical imaging of metabolites from corn leaf cross sections.

**Experimental**

**Chemicals**

See Supplementary Information.

**Analysis of standards**

Solutions of matrices (DAN, 9-AA, CHCA and DHB) were prepared at 10 mM in methanol. For water-soluble standards, a solution containing malic acid, glutamic acid, phosphoenolpyruvic acid and ascorbic acid at 10 mM each and UDP-glucose at 2.5 mM was prepared in water. For phospholipid standards, soy
phospholipid extract was dissolved at 2 mg/ml in chloroform. Each matrix solution was mixed 9 : 1 (v : v) with the analyte solution. The matrix-analyte mixture was then applied to a stainless steel MALDI target by spraying with a custom-made oscillating capillary nebulizer.[12] Compared with the dried-droplet method, this produced a very homogeneous sample coating, eliminating the ‘hot spot’ issue of dried-droplet preparation. A total of 200 μl of each matrix-analyte mixture was sprayed at a rate of 50 μl/min from a height of 9 mm with a nebulizing gas pressure of 40 psi. The spray device and slide were kept static during the spraying process, and this resulted in a coated area approximately 1.5 cm in diameter.

MALDI-MS analysis was performed on a linear ion trap-orbitrap hybrid mass spectrometer (MALDI LTQ-Orbitrap Discovery; Thermo Scientific, San Jose, CA, USA). The instrument has been modified to use an external Nd:YAG laser (UVFG; Elforlight, Ltd., Daventry, UK) operating at a repetition rate of 60 Hz. Spectra were collected using the orbitrap analyzer with a scan range of m/z 50–600 for LMW standards and m/z 500–1200 for phospholipid extract. Each spectrum was collected using 10 laser shots, and one spectrum was collected from each raster point. Data were acquired for a set of standard samples in triplicate in randomized sample order.

Laser energy was individually optimized for each matrix by rastering over an area of the sprayed sample and increasing the energy stepwise over the course of the scan. For each matrix, the laser pump diode energy was increased from 79% to 90% in increments of 0.5% and each energy setting was maintained for 1 min. On the basis of the measurements of the laser output energy, this range corresponds to approximately 0.2–18 μJ per pulse. Considering the estimated laser spot diameter of 50 μm, this yields laser fluences ranging from ~12 to ~900 mJ/cm². It is important to note that pulse energies were measured at the laser aperture, and some energy loss is inevitable at each of the beam steering and focusing elements. Therefore, the actual fluences at the sample surface are expected to be slightly lower.

After acquisition, time traces for the [M–H]⁻ species of each analyte were extracted and exported to Microsoft Excel. Signal levels for each analyte were averaged at each laser energy step and the signal level was plotted as a function of laser energy. These plots, shown in Supplemental Figure S1, were used to select the optimal laser energy for each matrix. As expected, metabolite standard signal levels were found to be heavily dependent among these three factors (signal intensity for non-labile compounds, signal intensity for labile compounds and reduction of matrix signals), the peak of the UDP-glucose signal was used to determine the ‘optimal’ laser fluence for each matrix, and this fluence was used to compare signal levels between matrices.

Maize leaf cross-sectional imaging

B73 inbred maize (Zea mays) seedlings were cultivated in soil in a greenhouse and harvested 14 days after germination. A transverse section of the third leaf approximately 15 cm from the stalk was cut, placed into a 10% w/v gelatin solution in a mold and floated on liquid nitrogen until the gelatin was nearly frozen solid. The embedded tissue was then transferred to a cryostat (Leica CM1850, Leica Microsystems, Buffalo Grove, IL, USA) at −20 °C and allowed to equilibrate at that temperature. Transverse tissue sections were cut at 10 μm thickness and collected using Cryo-Jane® adhesive tape (Leica Microsystems) to preserve tissue morphology. Tissue sections were left affixed to the tape windows, instead of thaw-mounting directly to glass or metal slides, to avoid any delocalization of soluble metabolites. Each tape window, with the tissue still affixed, was then attached to a glass slides for easier transport and storage, transported on dry ice and stored at −80 °C. Prior to analysis, an aluminum block (approx. 18 cm × 6 cm × 0.65 cm) was precooled to −80 °C. Slides holding tape and tissue sections were laid on this block and placed into a vacuum chamber, then dried at a pressure of ~50 mtorr for 90 min. Use of adhesive tape in cryosectioning, maintaining sample sections at ~80 °C and gradual drying/warming under vacuum provides tissue samples that are intact and completely dried for analysis and minimizes delocalization of water-soluble metabolites as a result of atmospheric condensation or sample thawing.

1,5-Diaminonaphthalene and 9-AA were applied by sublimation using a procedure similar to that described by Hankin et al.[13] By inserting a beam expander into the laser path,[14] the spot size at the sample surface was reduced to 20 μm, as estimated from ablation spots on the matrix-coated embedding material outside the analyzed tissue region. TunePlus and Xcalibur software (Thermo Scientific) were used to define the experimental parameters. Imaging data were acquired with a raster step size of 25 μm, 20 shots per scan and a scan window of m/z 100–1000. For imaging experiments, slightly higher laser energy was used due to energy loss at the beam expander. Laser energies were set at 83.5% and 85.0% for DAN and 9-AA, respectively. ImageQuest (Thermo Scientific) was used to generate MS images with a m/z window of ±0.005. Tentative assignments were made on the basis of accurate mass values.

Results and discussion

To avoid the ‘hot spot’ problem commonly seen with MALDI sample spotting, standard analysis was performed by spraying a mixture of standards and matrix using an oscillating capillary nebulizer device. A portion of the sprayed area was then scanned while the laser pulse energy was periodically increased to find the optimal laser energy for each matrix. As expected, metabolite standard signal levels were found to be heavily dependent upon laser fluence. Supplemental Figure S1 shows the intensity of each of the standard compounds and the predominant matrix ion ([M–H]⁻ for each case) with varying laser energy. In the case of DAN, all ion signals increase to a peak intensity in the 81–83% energy range (90–270 mJ/cm² fluence range), then decline at higher energies. With 9-AA, CHCA and DHB, signals for malic, glutamic and ascorbic acids increase across the range of laser fluences investigated. UDP-glucose and phosphoenolpyruvic acid, however, which contain labile bonds, exhibit a similar behavior regardless of matrix: Both signals peak at lower laser fluences and decline significantly beyond that, as the excess energy leads to in-source decay of the [M–H]⁻ species. In addition, excess laser energy gives rise to increased matrix background signals. Therefore, as a compromise among these three factors (signal intensity for non-labile compounds, signal intensity for labile compounds and reduction of matrix signals), the peak of the UDP-glucose signal was used to determine the ‘optimal’ laser fluence for each matrix, and this fluence was used to compare signal levels between matrices. These optimal values correspond to calculated fluences of ~90 mJ/cm² for DAN and CHCA, ~130 mJ/cm² for 9-AA and ~270 mJ/cm² for DHB.

A comparison of spectra obtained from each matrix-metabolite standard mixture at the optimal laser energy is presented in Fig. 1. Significant suppression of the matrix background signals is seen
Figure 1. MALDI-MS spectra of a metabolite standard mixture using several different matrices (a, DAN; b, 9-AA; c, CHCA; d, DHB). Spectra represent approximately 64 averaged scans at the optimal laser energy for UDP-glucose signal. Label of peaks are as follows: M, malic acid (m/z 133.014); G, glutamic acid (m/z 146.046); P, phosphoenolpyruvic acid (m/z 166.975); A, ascorbic acid (m/z 175.025); U, UDP-glucose (m/z 565.047); M0, P0 and U0 fragments of M, P and U, respectively; Aac, adducts of A and matrix; m or m0, matrix peaks; MOb, a malic acid-borate complex; MDB, a malic acid-DHB-borate complex and DB, a DHB-borate complex. A list of peak assignments is provided in Supplementary Table S1.

Figure 2. Comparison of metabolite ion signals and predominant matrix signal ([M – H]–) for each matrix at optimal laser energy. Values represent the average of three replicates and are shown normalized to the highest intensity for each metabolite. Error bars represent one standard deviation.

Diaminonaphthalene as small molecule MALDI matrix

for both DAN and 9-AA, relative to the matrix blank spectra (Supplemental Figs S2a, S2b). All analytes are detected as the [M – H]– species at some level with all four matrices (malic acid, m/z 133.014; glutamic acid, m/z 146.046; phosphoenolpyruvic acid, m/z 166.975; ascorbic acid, m/z 175.025; UDP-glucose, m/z 565.047), though with significant differences in signal intensity. Additional peaks detected at >5% relative abundance and identified on the basis of accurate mass measurements are listed in Supplemental Table 1. DAN and 9-AA both show appreciable signal for the [M – H2O – H]– fragments of malic acid and phosphoenolpyruvic acid. DAN also shows signal for adducts of DAN and ascorbic acid (m/z 315.098, [DAN + ascorbic acid – H2O – H]– and m/z 331.093, [DAN + ascorbic acid – 3H]–). The 9-AA spectrum contains several UDP-glucose fragments and phosphate-like signals that are suspected to arise from UDP-glucose, in line with previous observations that MALDI-MS using 9-AA as matrix leads to significant fragmentation of phosphorylated nucleotides. The CHCA spectrum is dominated by matrix-derived signals. The DHB spectrum contains matrix-derived peaks and a peak corresponding to a malic acid-DHB-borate cluster, similar to the borate complexes observed by Penn et al. For quantitative comparison of MALDI efficiency, [M – H]– ion signals of each analyte in Fig. 1 are compared on the basis of their absolute values, as shown in Fig. 2. Each signal is normalized to the highest value among the four matrices and represents the average of three non-consecutive acquisitions. DAN was found to provide significantly higher signal levels for malic, glutamic, phosphoenolpyruvic and ascorbic acids (11×, 30×, 3× and 4× higher than the next highest signal for the other tested matrices, respectively). However, 9-AA shows 2.5 times higher intensity for UDP-glucose than DAN, suggesting significant selectivity for different metabolite classes.

A comparison of spectra obtained from soy phospholipid extract using DAN and 9-AA is shown in Fig. 3. A laser optimization procedure similar to that used for small metabolites was performed for the phospholipid mixture. Phospholipid signals were found to plateau at intermediate laser energies (82.5% for DAN and 83.5% for 9-AA), with little difference in spectral profile with increasing energy from these values (data not shown). Therefore, the beginning of this signal plateau was chosen for comparison. The manufacturer-provided phospholipid content of the mixture is as follows: 24.0% phosphatidylcholine (PC), 18.6% phosphatidylethanolamine (PE), 11.5% phosphatidylinositol (PI), 4.3% phosphatidic acid (PA), 4.6% Lyso-PC and 37.0% unknown. Because of the quaternary amine functional group, PC is not differentially ionized whereas PI and PE and N-acylphosphatidylethanolamines. The spectra obtained using DAN as matrix provide a phospholipid profile closer to the specified composition, whereas 9-AA seems to preferentially ionize PI species while suppressing signal for other phospholipids. For example, the ratio of summed ion count for PE/PI/PA is 100 : 82 : 8.6 for DAN and 100 : 803 : 33 for 9-AA, whereas that provided by the manufacturer is 100 : 37 : 40. Phospholipids identified from the soy PL mixture are listed in Supplemental Table S2.
Encouraged by the promising results of small molecule analysis by DAN and 9-AA, we applied both for the chemical imaging of cross sections of maize seedling leaves. Figure 4 shows the chemical images of various small molecules from these leaf sections using DAN and 9-AA on adjacent sections. On the basis of accurate mass measurements, these signals were assigned to a wide range of metabolite classes, including amino acids such as asparagine and glutamic acid (4a, 4c), small organic acids such as malic and ascorbic acids (4b, 4d), flavonoid and flavonoid glycosides such as kaempferol and maysin (4e, 4g), benzoxazinoids such as HMBOA-glucose (4f) and sulfolipids such as sulfoquinovosyl diacylglycerol (34 : 3), (4 h).

A number of distinct distributions are observed for different metabolites, and several of these distributions correlate to tissue structural features. Asparagine (4a) is seen in localized clusters near the midrib section of the leaf. Malic acid (4b) and glutamic acid (4c) are detected more or less evenly throughout the interior leaf tissues. Ascorbic acid (4d) is detected both in interior and epidermal tissues, consistent with its multitude of metabolic roles in a wide range of cell types. Flavonoids and flavonoid glycosides (4e, 4g) are observed within the epidermal cell layers on the leaf exterior, in agreement with previous reports and their role in UV protection and plant defense. Interestingly, the flavonoid C-glycoside maysin (4g) is readily detected in the upper epidermis and largely absent in the lower epidermis. HMBOA-glucose (4f) is detected in clusters in the mesophyll between the peripheral vascular bundles, rather than within the vascular bundles themselves. SQDG (34 : 3), (4h) is detected in a series of rings along the length of the leaf, closely following the pattern of photosynthetic cells surrounding the vascular bundles. The vascular bundles are clearly visible as the ring centers, where no SQDG is detected. This distribution agrees with the known presence of SQDG in the membranes of photosynthetically active cells. For the reader’s reference, an optical microscope image showing a portion of a maize leaf section and a schematic

Figure 3. Spectra obtained from soy phospholipid extract using DAN (top) and 9-AA (bottom) as matrix. Spectral regions that are dominated by a particular phospholipid class are highlighted and labeled with the following class: PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; and NAPE, N-acylphosphatidylethanolamine. A list of peak assignments is presented in Supplementary Table S2.

Figure 4. Chemical images of corn leaf cross section with DAN and 9-AA as matrices. Images were generated using a mass window of ±0.005 Da. Brighter color indicates higher intensity. Scales range from zero to a maximum intensity indicated by the value at the bottom right of each image. Tentative assignments made on the basis of accurate mass values are as follows, all as [M–H]+ ions: (a) m/z 131.046, asparagine; (b) m/z 133.014, malic acid; (c) m/z 146.046, glutamic acid; (d) m/z 175.025, ascorbic acid; (e) m/z 285.040, C_{15}H_{10}O_{6}, flavonoid, for example, kaempferol; (f) m/z 356.099, HMBOA-glucose; (g) m/z 575.144, C_{15}H_{29}O_{14}, flavonoid glycoside, for example, maysin; and (h) m/z 815.504, SQDG (34 : 3).
Diaminonaphthalene as small molecule MALDI matrix

showing the structural features of the analyzed sections is provided in Supplemental Fig. S3.

As expected from the standard analysis, metabolite signal from tissue using DAN is generally higher than 9-AA, especially at low mass below m/z ~400, whereas 9-AA is slightly better for some compounds in high mass range above m/z ~400. Although many of the metabolite distributions (e.g., flavonoids and SQDG) can be explained from known functions or previously observed localizations, the biological implications of some (e.g., HMBOA-glucose) are not readily apparent and will require further investigation; nonetheless, these results demonstrate the ability of both matrices to ionize metabolites with a wide range of functionalities from tissue.

Conclusion

We have successfully demonstrated the application of DAN as a MALDI matrix for small molecule metabolites in both profiling and imaging. DAN was compared with three common MALDI matrices: 9-AA, CHCA and DHB. For the investigated compounds, DAN compares favorably or significantly exceeds the performance of the other tested matrices. Specifically, DAN shows superior ion signals for MW <~400 Da in both selected standards and some plant metabolites on cross-sectional leaf tissue. For medium molecular weight molecules (600 to ~1000 Da), 9-AA shows better performance in some compounds; however, DAN was found to provide a more representative characterization of phospholipid content for a plant-derived extract. We suggest that DAN, although previously used for imaging and analysis of lipids, is also useful for small metabolite analysis in negative mode, and that its utility for both low-mass analytes and larger lipids makes it appealing as a ‘universal’ matrix for metabolite imaging in the negative mode.

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References


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