

RESEARCH ARTICLE

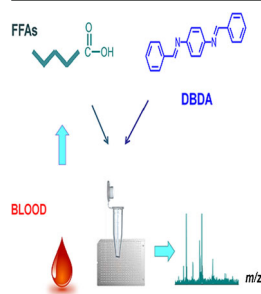
DBDA as a Novel Matrix for the Analyses of Small Molecules and Quantification of Fatty Acids by Negative Ion MALDI-TOF MS

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Abstract. Matrix interference ions in low mass range has always been a concern when using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) to analyze small molecules (<500 Da). In this work, a novel matrix, *N*1,*N*4-dibenzylidenebenzene-1,4-diamine (DBDA) was synthesized for the analyses of small molecules by negative ion MALDI-TOF MS. Notably, only neat ions ($[M-H]^-$) of fatty acids without matrix interference appeared in the mass spectra and the limit of detection (LOD) reached 0.3 fmol. DBDA also has great performance towards other small molecules such as amino acids, peptides, and nucleotide. Furthermore, with this novel matrix, the free fatty acids in serum were quantitatively analyzed based on the correlation curves with correlation coefficient of 0.99. In

addition, UV-Vis experiments and molecular orbital calculations were performed to explore mechanism about DBDA used as matrix in the negative ion mode. The present work shows that the DBDA matrix is a highly sensitive matrix with few interference ions for analysis of small molecules. Meanwhile, DBDA is able to precisely quantify the fatty acids in real biological samples.

Keywords: MALDI-TOF MS, Matrix, Small molecules, Fatty acids, Quantification, Negative ion mode

Received: 29 May 2017/Revised: 18 December 2017/Accepted: 21 December 2017/Published Online: 18 January 2018

Introduction

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is a powerful tool for the characterization of large biomolecules [1–5], such as proteins, peptides, oligosaccharides, oligonucleotides, as well as synthetic polymers because of its high throughput, soft ionization, high salt tolerance, and high sensitivity. Nevertheless, the analysis of small molecules (<500 Da) by MALDI-

TOF MS is still limited because the commonly used organic matrices, such as *o*-cyano-4-hydroxycinnamic acid (CHCA) and 2,5-dihydroxybenzoic acid (DHB), always produce strong matrix-associated ions in the low-mass range [6, 7], which interfere or even suppress the analyte signals.

To solve this problem, efforts have been made to reduce the interference ions in low-mass range. A number of inorganic matrices, such as porous silicon [8], carbon based materials (graphite, graphene, carbon nanotubes, carbon nanodots, etc.) [9–13], and other metal nanoparticles [14] are reported continuously. However, the synthesis of these materials could be complicated or expensive. In addition, several organic matrices with less interference ions have been developed for small molecules analysis. For instance, 9-aminoacridine (9AA) [15], *N*-(1-naphthyl) ethylenediamine dinitrate [16], *N*-(1-naphthyl) ethylenediamine dihydrochloride (NEDC) [17], and 1,5-

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s13361-017-1881-y>) contains supplementary material, which is available to authorized users.

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diaminonaphthalene hydrochloride [18] were reported to be efficient for analysis of various small molecules. Furthermore, superbasic matrices such as 1,8-bis(dimethylamino)naphthalene (DMAN) [19], 1,8-di(piperidinyl)naphthalene (DPN) [20] optimized from DMAN, and azahelicene superbases [21] were reported by Svatoš et al. The authors suggested that superbasic matrices captured the proton from acidic analyte in solution according to Brønsted-Lowry acid base theory during sample preparation. They called this ionization process “matrix-assisted ionization/laser desorption” (MAILD) [22]. Those previous researches indicated that the matrices function depends on both properties of analytes and their own, and additional matrices for MALDI-MS analysis of small molecules are still needed on one hand to enhance analytical efficiency and sensitivity and on the other hand to understand ionization mechanism of MALDI-MS.

In this study, we designed and synthesized six Schiff base compounds and screened out a most efficient matrix, N,N'-dibenzylidenebenzene-1,4-diamine (DBDA) for analysis of small molecules. The performance of DBDA towards various small molecules (amino acids, peptide, nucleotide, fatty acid) was demonstrated by comparison with 9AA, DMAN, and NEDC in the negative ion mode.

Free fatty acids (FFAs), either saturated or unsaturated aliphatic carboxylic acids, serve as the raw materials of lipoprotein, grease, phospholipid, and glycolipid or functional compounds for human health or diseases [23–26]. Hence, with DBDA in hand, we further explored its application in analysis of FFAs in serum. Under optimized conditions, serum FFAs were accurately quantified using DBDA matrix. Our results demonstrated that DBDA is an excellent matrix for small molecules, particularly for the analysis of fatty acids FFAs. The possible mechanisms related to great performance of DBDA were discussed based on experiments and theoretical calculation.

Experimental

Chemicals and Reagents

Amino acids, glutathione, 5'-adenylic acid, standard FFAs including myristic acid (C14:0), palmitic acid (C16:0), heptadecenoic acid (C17:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), arachidic acid (C20:0), arachidonic acid (C20:4), behenic acid (C22:0), docosahexaenoic acid (C22:6), 9AA, DMAN, NEDC, 1,4-diaminobenzene, and benzaldehyde were purchased from J&K Chemical (Beijing, China). The solvents, including chloroform, methanol, isopropanol, acetonitrile, ethanol, all analytical grade, were purchased from Beijing Chemicals (Beijing, China). The serum samples were obtained from Dr. Yang Xu at the Jilin University. The water was made by Milli-Q system.

Synthesis of Schiff Base Compounds

The DBDA was synthesized by the Schiff base reaction (Supplementary Scheme S1). Briefly, 1,4-diaminobenzene

and benzaldehyde were mixed at the mole ratio of 1:3 in anhydrous ethanol and the obtained solution was refluxed for 6 h at 90 °C. Then the mixture was treated with suction filtration and recrystallization using anhydrous ethanol three times (other Schiff base compounds were carried out using a method similar to that used for DBDA); ¹H NMR (500 MHz, DMSO) and (ppm); 8.70 (s, 2H), 7.98–7.94 (m, 4H), 7.54 (dd, J = 5.1, 2.1 Hz, 6H), 7.37 (s, 4H) (Supplementary Figure S1).

UV-Vis Analysis

The Schiff base compounds were prepared at concentration of 4.5 µg/mL in methanol/chloroform (1:1, v/v), and 1 mL of the matrix solution was transferred to cuvette for UV-Vis absorption. A cuvette equipped with 1 mL of methanol/chloroform (1:1, v/v) was used as the reference.

Matrix and Sample Preparation

The matrix DBDA was prepared at 10 mg/mL in methanol/chloroform (1:1, v/v). 9AA and NEDC were dissolved in isopropanol/acetonitrile (1:1, v/v) and water/acetonitrile (3:7, v/v) respectively, both at 10 mg/mL. DMNA was prepared in ethanol at the same concentration as the analytes. The stored solutions of pure FFAs were prepared at 5 mM in chloroform. The amino acids, glutathione, and 5'-adenylic acid were dissolved in water at 1 mM. For MALDI-MS analysis, the analyte and matrix solutions were mixed in equal volumes, and 0.4 mL of the obtained solution was spotted on target plate and dried at room temperature. For the quantitative analysis of FFAs in serum, the “seeds” sample deposition method was adopted [27]. First, matrix solution was diluted five times, and 0.4 µL matrix was placed on the target as seeds. After solvent evaporation, the 2 µL sample solution was mixed with 2 µL matrix solution. Subsequently, 0.4 µL of the mixed solution was placed on the pre-deposited crystal and followed by air drying before MALDI-MS analysis.

Crude Extract of Serum FFAs

Three hundred µL of freshly thawed serum solution was pipetted into a centrifuge tube. Then 1 mL of methanol was added, and the mixture was vortexed for perfect mixing and stored at –18 °C overnight. The overall solution was centrifuged at 19,000 rpm for 30 min at 4 °C. Then the supernatant was transferred into another centrifuge tube, to which 1 mL of n-hexane and 1 mL of Milli-Q water were added. The resultant mixture was vortexed for 5 min and centrifuged at 5000 rpm for 10 min. Finally, the hexane layer with the serum FFAs was pipetted into another tube, air-dried, and then stored at –18 °C.

Quantification and Method Validation

Heptadecenoic acid (C17:1) was used as the internal standard (IS) to improve quantitative accuracy [28]. Each standard FFA was diluted to a series of concentrations and the FFAs solutions were mixed with IS (40 µg/mL). The obtained mixtures were analyzed by MALDI-MS. The calibration curves of each FFAs were constructed by the intensity ratios of FFAs to IS versus the

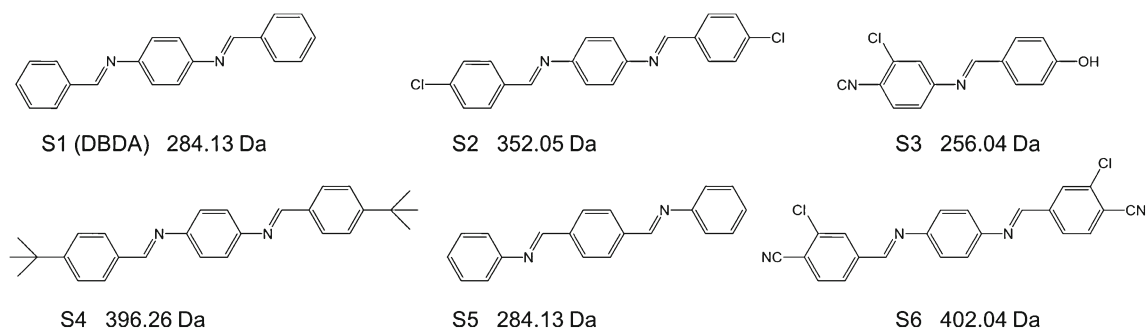


Figure 1. The structures and molecular weights of Schiff base compounds investigated in this study

concentrations of respective FAs. For the quantification of FFAs in serum, crude extract of serum FFAs were dissolved in 150 μ L chloroform containing 40 μ g/mL of IS and analyzed by MALDI-MS. The concentrations of serum FFAs were calculated based on the calibration curve equations of each standard FAs.

The reproducibility was estimated by detection of the same samples that were handled using the same procedure. The relative standard deviations (RSD) were acquired from six parallel experiments. The limit of detection (LOD) was calculated according to the lowest concentration of analyte detected as $[M-H]^-$ with signal-to-noise ratio (S/N) ≥ 3 . The spike-and-recovery experiments were carried out as follows: three serum samples were randomly selected and each was divided in duplicate; one portion of each sample was mixed with IS (20 μ g/mL). Another portion of three samples were spiked with different concentration of FAs and fixed concentration of IS (20 μ g/mL). (Three different concentrations of the spiked FAs are listed below: sample 1: C16:0, C18:0, C18:1, C18:2, and C20:4 with the concentrations of 10 μ g/mL, C22:0 and C22:6 with the concentration of 5 μ g/mL;

sample 2: C16:0, C18:0, C18:1, C18:2, and C20:4 with the concentrations of 50 μ g/mL, C22:0 and C22:6 with the concentration of 10 μ g/mL; sample 3: C16:0, C18:0, C18:1, C18:2, and C20:4 with the concentrations of 100 μ g/mL, C22:0 and C22:6 with the concentration of 20 μ g/mL). Then the FAs were extracted from the serum samples according to the above-mentioned method.

Molecular Orbital Calculations Approach

The Gaussian 09 program was used with the B3LYP functional density approach at the B3LYP/6-311+G (d,p) level of theory to calculate the proton affinities, ionization energies, and electron affinities of DBDA, 9AA, DMAN, CHCA, and DHB in the gas phase. The basis sets have been proven to be able to obtain similar thermochemical properties of traditional matrices with the individual experimental value [29].

Instrumentation

The MALDI-TOF mass spectra were recorded on a Bruker Autoflex speed TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Its laser device is a pulsed

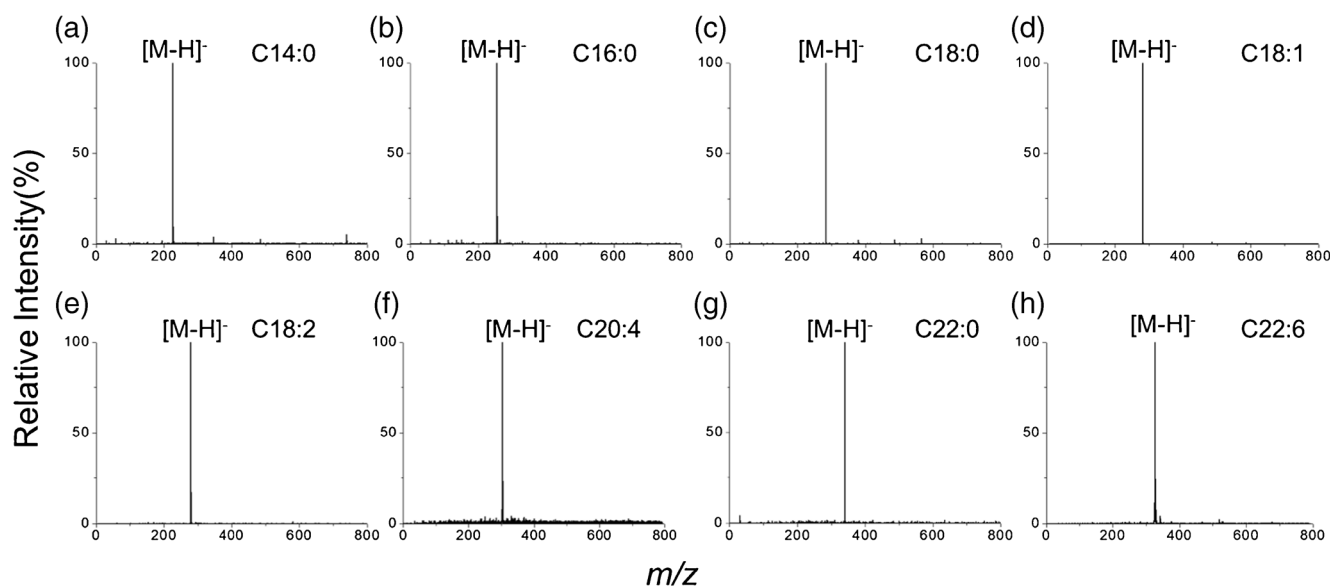


Figure 2. The MALDI-MS spectra of saturated and unsaturated FAs using DBDA as matrix in the negative ion mode; (a) C14:0, (b) C16:0, (c) C18:0, (d) C18:1, (e) C18:2, (f) C20:4, (g) C22:0, (h) C22:6

Table 1. The LOD of FAs Using DBDA as Matrix by MALDI-MS in the Negative Ion Mode

FAs	C14:0	C16:0	C18:0	C18:1	C18:2	C20:4	C22:0	C22:6
LOD/fmol	0.18	0.31	0.28	0.28	0.28	0.66	0.23	0.61

Nd:YAG with the wavelength of 355 nm. A ground steel sample target (Bruker Daltonics, Bremen, Germany) with 384 spots was used. All analytes mass spectra were obtained in negative ion mode with the delayed extraction time of 150 ns and the acceleration voltage of 19 kV. All mass spectra were processed by the Flex Analysis 3.0 software (Bruker Daltonics). The UV spectra were measured using a UV-2550 spectrophotometer (SHIMADZU, Kyoto, Japan).

Results and Discussion

Screening of Schiff Base Compounds for Great Performance Matrix

Six Schiff base compounds (S1–S6) with conjugated structure were synthesized and tested to see whether they are potential MALDI matrices for small molecules in the negative ion mode. Their structures are listed in Figure 1. According to the photoionization mechanism [30], electronic excitation of matrices is the initial ionization step; thus it is important that MALDI matrices have strong ultraviolet absorption at the instrument's laser wavelength

(355 nm). Hence, the UV-Vis absorbances of six compounds were tested first and the results are shown in Supplementary Figure S2. DBDA has maximal absorption at 355 nm, and it has a large mole absorption coefficient of $\epsilon = 2.3 \times 10^4 \text{ M}^{-1}$. The absorption of compound S4 is slightly lower than that of DBDA. Next, the mixtures of saturated FAs (C14:0, C16:0, C18:0, C20:0, C22:0) were analyzed using synthesized Schiff base compounds as matrices. Interestingly, the five FAs were detected with significantly high S/N (>1000) in the negative ion mode using DBDA (Supplementary Table S1). With the compounds S2 and S4 (analogues of DBDA), as matrices, five FAs were detected but with lower S/N than DBDA. Moreover, the mass spectrum of DBDA is very clean in positive ion mode of MALDI-MS (Supplementary Figure S3). This is attributed to the strong ability of DBDA to seize a proton. With this great performance matrix DBDA, eight saturated and unsaturated FAs were individually analyzed (Figure 2) and the limit of detection (LOD) of these FAs were obtained by diluting gradually. As shown in Figure 2, hardly any of the matrix-related ions were observed in the spectra. There are only deprotonated ions of FAs ($[\text{C14:0-H}]^-$, $m/z = 227.2$; $[\text{C16:0-H}]^-$, $m/z = 255.2$; $[\text{C18:0-H}]^-$, $m/z = 283.3$; $[\text{C18:1-H}]^-$, $m/z = 281.2$; $[\text{C18:2-H}]^-$, $m/z = 279.2$; $[\text{C20:4-H}]^-$, $m/z = 303.2$; $[\text{C22:0-H}]^-$, $m/z = 339.3$; $[\text{C22:6-H}]^-$, $m/z = 327.2$) in the mass spectra. Also, FAs could be detected in the 10th femtomole range using DBDA as MALDI matrix (Table 1). These results demonstrated that DBDA is a potential matrix for small molecules. So DBDA was selected to following experiments.

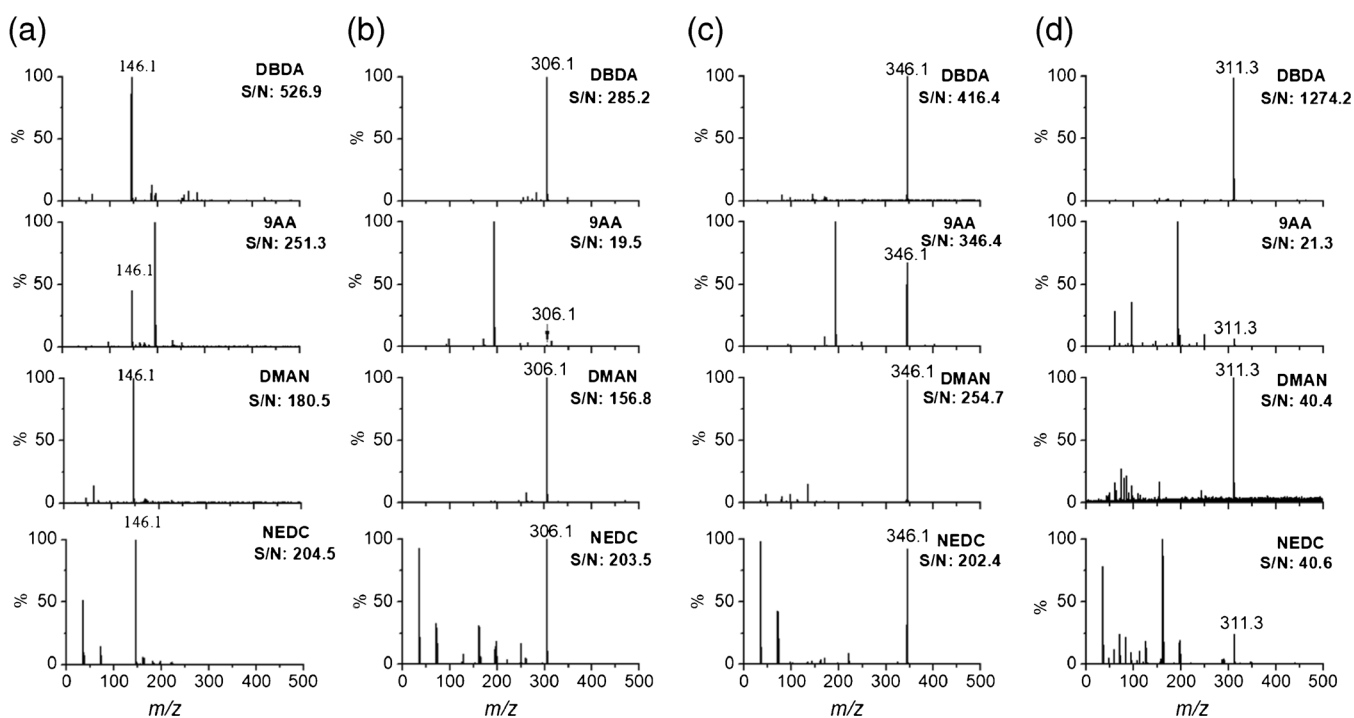


Figure 3. The MALDI-MS spectra of (a) glutamic acid, (b) glutathione, (c) 5'-adenylic acid, and (d) C20:0 (200 pmol on plate each) using different matrices; DBDA, 9AA, DMAN, and NEDC

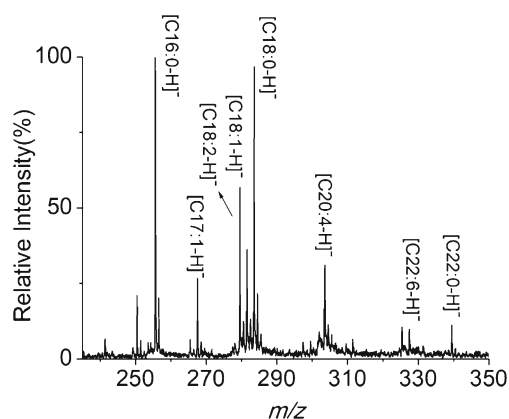


Figure 4. The MALDI-MS spectrum of serum FFAs including IS using DBDA as matrix in the negative ion mode

Evaluate the Performance of DBDA for Small Molecules

To evaluate the versatility of DBDA to various small molecules, three amino acids (glutamic acid, phenylalanine, lysine), glutathione, 5'-adenylic acid, and C20:0 were analyzed by comparison with the commonly used matrix 9AA [15, 31], superbasic matrix DMAN [19, 22], and organic salts matrix NEDC [17], which are reported for the analysis of metabolites. The representative spectra of acidic analytes are shown in Figure 3; using DBDA, glutamic acid, glutathione, 5'-adenylic acid, especially the C20:1, were detected with higher S/N than the results that using the 9AA, DMAN, and NEDC as matrices. Also, there are hardly any matrix interferences in mass spectra using DBDA matrix. 9AA and NEDC produced several matrix-related ions; particularly the ion of 9AA was always detected as base peak in the mass spectra. Additionally, the non-acidic amino acids phenylalanine and lysine were analyzed using these four matrices (Supplementary Figure S4). We found that DBDA has the greatest ionization efficiency for phenylalanine and lysine compared with 9AA, DMAN, and NEDC. These results

indicated that DBDA is a promising matrix for small molecules not only for acidic compounds.

Quantification of Serum FFAs Using DBDA Matrix

In order to explore the application of DBDA for real world sample, DBDA matrix was used for the analysis of FFAs in serum. The serum sample is simply pretreated and analyzed by MALDI-MS using DBDA matrix in the negative ion mode. Seven FFAs, including C16:0, C18:0, C18:1, C18:2, C20:4, C22:0, and C22:6 can be detected clearly. These seven FFAs were further quantified. To eliminate the effect of various factors on the detection and improve accuracy and reproducibility of the analysis, C17:1 was used as the IS based on the following two reasons; first, there is no C17:1 in serum, thus its content is completely determined by the amount added. Second, its property and molecular weight are similar to serum FFAs. Hence, the crude FFAs solution containing 40 $\mu\text{g/mL}$ IS was mixed with DBDA and analyzed by MALDI-MS. The representative mass spectrum is shown in Figure 4. During the analysis, two sample preparation methods, dried-droplet and “seeds”, were compared to improve the reproducibility. As shown in Figure 5, the MALDI-MS image which used the method of “seeds” greatly enhanced the crystal uniformity and therefore improved the reproducibility of the quantitative analysis. Afterwards, the standard curves of these seven FFAs were constructed based on the intensity ratio of the individual standard FFAs to the IS versus their respective concentration, and the corresponding equations are listed in Table 2. With our method, the standard curves were obtained with correlation coefficient $R^2 > 0.99$. The linear dynamic ranges are more than two orders of magnitude and the relative standard deviation (RSD) of individual FFAs are lower than 15% (Table 2). Based on the equations of each FFAs, the concentrations of FFAs in the five different serum samples were acquired and are listed in Supplementary Table S2. To further validate this method, the spike-and-recovery experiments were performed (details were given in the Experimental section) and recoveries of each FFAs ranged from 87.2% to 108% (Supplementary Table S2). These results indicated that using

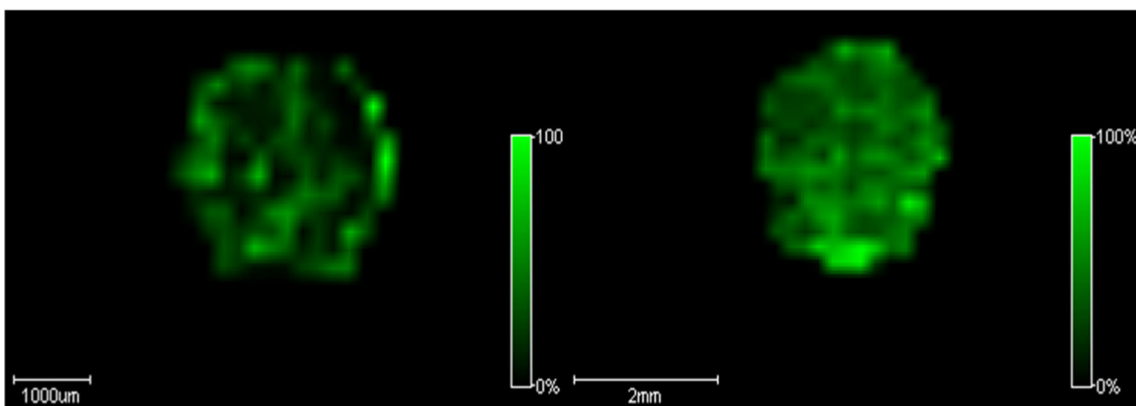


Figure 5. The MALDI-MS images of C16:0 using different sample preparation methods in the negative ion mode; dried-droplet (left) and “seeds” (right)

Table 2. Standard Curve Equation, Correlation Coefficient (R^2), Linear Range, and Relative Standard Deviation (RSD) of Each FAs

FAs	Standard curve equation (n = 6)	R^2	Linear range ($\mu\text{g/mL}$)	RSD (%) (n = 6)		
				10 $\mu\text{g/mL}$	50 $\mu\text{g/mL}$	100 $\mu\text{g/mL}$
C16:0	$y=0.0530x+0.7556$	0.995	1–650	13	9	9
C18:0	$y=0.0717x+1.4163$	0.994	10–650	10	13	12
C18:1	$y=0.0403x+0.0475$	0.9996	5–800	10	12	9
C18:2	$y=0.0502x+0.1848$	0.9993	5–750	12	11	13
C20:4	$y=0.0137x+0.0615$	0.994	1–550	11	12	15
C22:0	$y=0.1411x-0.3940$	0.9991	5–650	10 ^a	12 ^b	13 ^c
C22:6	$y=0.0736x-0.0637$	0.995	1–350	10 ^a	13 ^b	15 ^c

The “a”, “b”, and “c” represent concentration 5 $\mu\text{g/mL}$, 10 $\mu\text{g/mL}$ and 20 $\mu\text{g/mL}$, respectively

DBDA matrix and with our optimized method, the concentrations of the FFAs in serum could be accurately quantified.

Possible Related Mechanism

Using superbasic matrix, ionization of acidic analytes occurs in the solution phase, according to the acid-base theory developed by Brønsted-Lowry [22]. The analyte signal is highest when the matrix and analyte concentration is at mole ratio of 1:1. This ionization model was denoted as “matrix-assisted ionization/laser desorption” (MAILD). In order to explore the ionization mechanism of DBDA, we compared the UV-Vis absorption curve of DBDA with the mixture of C16:0 and DBDA at mole ratio of 1:1 in the same condition. However, we found that addition of C16:0 to DBDA solution could not change the UV-Vis absorption of DBDA (Supplementary Figure S5). From this experiment result, we speculated that the ionization step occurs in the gas phase but not in the solution phase. Furthermore, we analyzed C16:0 by mixing it with different concentrations of DBDA by MALDI-MS in the negative ion mode, and the results are shown in Supplementary Figure S6. When the mole ratio of DBDA/C16:0 increased, the signal of C16:0 rose continuously. It suggests that the ionization mechanism for DBDA is based on the conventional model “MALDI” and is different from DMAN.

We compared the gas-phase parameters of DBDA related with ionization with the commonly used matrix 9AA and superbasic matrix DMAN. Molecular orbital calculations were carried out to obtain proton affinities (PA), ionization energies (IE), and electron affinities (EA) of the matrices in the gas phase. Also, the parameters of commonly used matrices CHCA and DHB in the positive mode were calculated. As shown in

Table 3. Proton Affinities (PA), Ionization Energies (IE), Electron Affinities (EA), and (IE-EA) of Five Matrices; DBDA, 9AA, DMAN, CHCA, and DHB

Matrices	PA (kJ/mol)	IE (eV)	EA (eV)	IE-EA (eV)
DBDA	976	6.87	1.35	5.52
9AA	1026	6.93	0.80	6.13
DMAN	1029	6.28	-0.14	6.42
CHCA	855	8.36	1.65	6.71
DHB	852	8.13	0.29	7.84

Table 3, the PA of DBDA, 9AA, and DMAN are obviously stronger than the acidic matrices CHCA and DHB. In other words, alkaline matrices combine with protons more easily than acidic matrices in the gas phase. For DBDA, it captured the proton of FAs and was itself protonated, and the deprotonated FAs was detected in negative ion mode. FAs could not be detected using acidic matrices CHCA and DHB whether in the positive ion mode or in the negative ion mode [22]. In addition, DBDA is high sensitive for the analysis of FAs (LOD reached 0.3 fmol), which may be attributed to its strong ionization capacity. The smaller difference between the IE and the EA of each matrix, the easier primary ionization step could occur in MALDI MS [29]. The (IE-EA) of DBDA is obviously lower than other matrices, which indicated that DBDA is easier to ionize and transfer energy more effectively; this maybe explain why using DBDA to detect FAs can obtain a lower detection limit.

Conclusions

This work demonstrated that DBDA as a novel matrix can commendably detect the small molecules (amino acids, peptides, nucleotide, FAs) with hardly any matrix interference by MALDI-MS in the negative ion mode. Using DBDA matrix, the FAs were analyzed with low LOD of 0.3 fmol. Furthermore, this study also indicated that DBDA can be applied for rapid and accurate quantitative analysis of serum FFAs by negative ion MALDI-MS. The strong UV absorption at instrument laser wavelength (355 nm) and the great ionization capability make DBDA perform an excellent matrix for the analysis of small molecules. Further applications of DBDA for metabolomics are currently in progress in our laboratory.

Acknowledgments

This work was financially supported by the National Natural Science Foundation of China (51273080, 21675060, and 51203153) and International Collaboration Program of Jilin province (20160414010GH).

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