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Furoic and mefenamic acids as new matrices for matrix assisted laser desorption/ionization-(MALDI)-mass spectrometry

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ABSTRACT

The present study introduces two novel organic matrices for matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) for the analysis of small molecules. The first matrix is "2-amino-4,5-diphenylfuran-3-carboxylic acid" (also called furoic acid, FA) which was synthesized and then characterized by ultraviolet (UV), infrared (FTIR), nuclear magnetic resonance NMR (^{1}H and ^{13}C) and mass spectrometry. The compound has organic semiconductor properties and exhibits intense UV-absorption which is suitable for the UV-MALDI laser (N_2 laser, 337 nm). The second matrix is mefenamic acid (MA). The two matrices can be successfully applied for various classes of compounds including adenosine-5′-triphosphate (ATP, 0.5 μL(10.0 nmol)), spectinomycin (spect, 0.5 μL(14.0 nmol)), glutathione (GSH, 0.5 μL (9.0 nmol)), sulfamethazole (SMT, 0.5 μL(2.0 nmol)) and mixture of peptides gramicidin D (GD, 0.5 μL (9.0 nmol)). The two matrices can effectively absorb the laser energy, resulting in excellent desorption/ionization of small molecules. The new matrices offer a significant enhancement of ionization, less fragmentation, few interferences, nice reproducibility, and excellent stability under vacuum. Theoretical calculations of the physical parameters demonstrated increase in polarizability, molar volume and refractivity than the conventional organic matrices which can effectively enhance the proton transfer reactions between the matrices with the analyte molecules. While the reduction in density, surface tension and index of refraction can enhance homogeneity between the two new matrices with the analytes. Due to the sublimation energy of mefenamic acid is (1.2 times) higher than that of the DHB, it is more stable to be used in the vacuum.

1. Introduction

Matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) was discovered to solve the problems of fragmentation from direct laser desorption/ionization mass spectrometry (LDI-MS) for ionization of large biomolecules [1,2]. MALDI-MS is a simple and rapid analytical tool which can analyze nonvolatile and thermal stable molecules. Matrix is the most important/significant factor for the success of MALDI-MS analysis of various types of compounds. The function of matrix is to absorb laser radiation and to generate ions for analytes via proton transfer reactions. The matrix is vaporized or desorbed by laser to carry the intact analyte molecules into the gas phase prior to ionization. Selection the matrix is depending on the types of the laser which is used for desorption/ionization of analytes. To date, several types of lasers including nitrogen (337 nm), Nd:YAG (λ=355 or 266 nm), excimer or CO_2 lasers have been successfully applied for MALDI-MS analysis for various compounds [3].

To date, the matrices applied in MALDI-MS typically can be classified into two types: (1) organic matrices such as 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid, SA) [2], α-cyano-4-hydroxycinnamic acid (alpha-cyano or alpha-matrix, CHCA) [4], 2,5-dihydroxybenzoic acid (2,5-DHB) [5], their ionic liquid salts [6,7], and other organic matrices [8–11], (2) inorganic matrices [12–34]. In order to serve as a feasible or efficient MALDI matrix, the organic compounds should have the following features: (1) excellent absorption capability for laser radiation; (2) assistance in forming analyte ions after absorbing laser radiation; (3) good stability under vacuum; (4) isolation of the generated ions and prevent forming analyte clusters; (5) forming homogenous crystals with analytes; and (6) reduction of interferences upon ionization/desorption.

Small molecules (< 500 Da) analysis is a challenge in MALDI-MS due to the intensive background interferences of conventional

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organic matrices [2,4,5] which can produce cluster ions, suppress and overlap with the analyte signals. Therefore, matrix-free and material-enhanced methods, such desorption ionization on silicon (DIOS) and nanoparticles were extensively proposed [12–34]. However, seeking new organic matrices [35–38] are still required because of the convenience, easy accessibility, high sensitivity, and excellent performance in MALDI-MS analysis/imaging [39–41].

We introduce two novel organic matrices (furoic acid, FA and mefenamic acid, MA) for MALDI-MS analysis on small molecules. The furan derivatives are typically organic semiconductors which are common used as active components in electronics and optoelectronics. Due to their flexibility, low cost and ease of production, they represent a valid alternative to the conventional inorganic semiconductors [42]. For the first time, they were successfully applied as effectively organic matrices in the UV-MALDI-MS. The two matrices have demonstrated excellent applicability to analyze various analytes such as adenosine-5′-triphosphate (ATP), spectinomycin (Spect), glutathione (GSH), sulfamethazole (SMT) and peptides (Gramicidin D). These compounds were used as model compounds to confirm the nice capability of the two new matrices to serve as effective matrices in MALDI-MS.

2. Materials and methods

2.1. Materials

Adenosin-5′-triphosphate, gramicidin D, spectinomycin, benzoin, malononitrile, dimethyl formamide (DMF), diethylamine (Et_2NH), and nBuOH were purchased from Aldrich Chemical Co (Milwaukee, WI, USA). 2,5-dihydroxybenzoic acid (2,5-DHB), α-cyano-4-hydroxy cinnamic acid (4-CHCA), ethanol (EtOH), trifluoracetic acid (TFA), sulfamethazole (SMT), and glutathione (GSH) were purchased from Sigma-Aldrich. All chemicals were used directly without purification.

2.2. Instruments

2.2.1. Matrix assisted laser desorption/ionization (MALDI-TOF)

The MALDI-TOF-MS analyses were performed by employing positive ion mode on a time-of-flight mass spectrometer (Microflex, Daltonics Bruker, Bremen) with a 1.25 m flight tube. Desorption/ionization was obtained by using a 337 nm nitrogen laser with a 3 ns pulse width. Available accelerating potential is in the range of +20 kV. The spectra presented in this paper represent averages of 200 laser shots. Laser power was adjusted to slightly 10% above the threshold to obtain good resolution and signal-to-noise ratios. The data repeated at least three times to confirm repeatability.

2.2.2. Electrospray ionization-mass spectrometry (ESI-MS)

ESI-MS measurements were performed using an ion trap mass spectrometer (Finnigan LCQ-Advantages, Thermo, San Jose, CA, USA). A micro syringe pump (Harvard Apparatus, Edenbridge, Great Britain) was used for injecting sample solution. All mass spectra were obtained in the positive ion mode and the mass spectra collected in the positive mode were represented as ESI (+)-MS. The ion trap analyzer was operated at a pressure of ~1.5×10^{-5} Torr. The capillary temperature was 200 °C and the ESI spray needle voltage was 4.50 V. A tube lens offset voltage of 80.0 V and capillary voltage of 9.59 V were used to obtain ESI mass spectra. The sheath gas flow rate during the experiments was 18.90 mL/min.

The absorption spectra of all compounds were measured using an UV–vis spectrometer (U3501, Hitachi, Japan) from 300–700 nm. Transmission Infrared spectra were obtained using KBr discs (4000–400 cm^{-1}) on a FT-IR (Perkin Elemer 8300). NMR spectra were obtained using Bruker 300, Bremen, Germany. The structure and parameters calculations were measured using software of "ACD-Program V12, http://www.acdlabs.com/home/". Spectra are draw using Origin program V6.0 and fit as Gaussian curves.

3. Experimental section

3.1. Synthesis of 2-amino-4,5-diphenylfuran-3-carbonitrile

The 2-amino-4,5-diphenylfuran-3-carbonitrile was synthesized from benzoin through modifications of procedures in reference [43] as described in Scheme S1A. Briefly, diethylamine (13.8 g) was added dropwise over a period of 30 min to a mixture of benzoin (10.0 g, 47.2 mmol) and malononitrile (3.8 g, 57.6 mmol) in dimethyl formamide (DMF, 30.0 mL) at 0 °C (the reaction temperature should not exceed 40 °C). After that, the resulting mixture was stirred at room temperature for 16 h and then H_2O (100.0 mL) was added. The resulting precipitate was filtered, washed with a sufficient amount of H_2O, then n-hexane and dried. The solid was recrystallized from EtOH several times to form yellowish-brown solid products. The ultraviolet spectrum shown in Fig. S1(a), supporting file shows a peak at 325 nm. ESI(+)-MS spectrum (Fig. S1(b)) shows peaks at *m/z*: 261.1, 244.0, 218.1, and 234.1 which can be assigned as $[M+H]^+$, $[M+H–NH_3]^+$, $[M+H–NH_3–CN]^+$, and $[M–HCN]^+$, respectively. ^{1}HNMR (practical and stimulated spectra were shown in Fig. S1, 300 MHz, $CDCl_3$): $d=7.4–7.3$ (m, 8H), 7.2–7.1 (m, 2H), 5.2 (br, 2H). FT-IR in Fig. S1(d) shows peaks at wavenumber cm^{-1}: 3432–3120 (triplet, NH_2); 3062 (CH, aromatic); 2807 (CH, aliphatic); 2300(C≡N); and 1110(C–O).

3.2. Synthesis of 2-amino-4,5-diphenylfuran-3-carboxylic acid "Furoic acid, FA"

3.0 g of 2-amino-4,5-diphenylfuran-3-carbonitrile was dissolve into 25.0 mL of methanol, then it reflux with 2.0 mL of HCl (37%) for 5 h. The reaction container was cooled and the precipitates were washed with deionized water for three times. The precipitate was recrystalized for three times with ethanol. The yield of the reaction was about 96%. The ultraviolet spectrum in Fig. S2 (a) shows a peak about 335 nm. The MALDI(+)-MS spectrum (Fig. S2(b)) shows peaks at *m/z* 581.0, 557.8, 540.0, 522.0, 302.0, 279.4, 260.3 Da which can be assigned as $[2FA+Na]^+$, $[2FA+H]^+$, $[2FA–H_2O+H]^+$, $[2FA–2H_2O+H]^+$, $[FA+Na]^+$, $[FA+H]^+$ and $[FA–H_2O+H]^+$, respectively. ^{1}HNMR (Fig. S2(c), 300 MHz, $CDCl_3$): $d=10.0$ (s, H), 7.1–7.4 (m, 10 H), 8.0 (S, 1H), 5.2 (br, 2H).^{13}C NMR (300 MHz, $CDCl_3$) 31.1(s, 1C), 73.5 (s,1C), 125.4–129.7(m,12C), 207.3(s,1C), 161.9(s,1C), 140.0(s,1C). FT-IR spectrum in Fig.S2(d) shows several function groups at wavenumber 3500 and 1750 cm^{-1} which were corresponding to O–H and C=O, respectively.

3.3. Matrix and samples preparation for MALDI-MS analysis

3.3.1. Matrix preparation

The conventional matrices (2,5-DHB and CHCA) were prepared according to the preparation procedures in literature [7,8]. 2-amino-4,5-diphenylfuran-3-carboxylic acid (furoic acid, FA, 50 mM) and mefenamic acid (MA, 50 mM) were prepared in aqueous methanol (50:50) with 0.1% TFA.

3.3.2. Sample preparation

Standard sample preparation was performed by mixing appropriate volumes of matrix and analyte solutions (1:1) with molar ratio 1–10^3. The dry-droplet technique was used for spotting all analytes onto the MALDI target plates. Briefly, 0.5 μL of different

matrixes (50 mM) and 0.5 μL of each analyte (Adenosine-5′-triphosphate, ATP, 0.5 μL(10 nmol)), spectinomycin (0.5 μL, 0.5 nmol), glutathione (0.5 μL, 9 nmol), sulfamethazole (0.5 μL, 2 nmol) and peptide "gramicidin D" (0.5 μL, 9 nmol) are mixed together in an eppendorf tube prior to spotting onto the MALDI plates. The MALDI-MS analysis was repeated in triplicates to obtain reproductive results.

4. Results and discussion

Prior to MALDI-MS analysis, the concentrations of analytes were diluted in the matrices at typical mass or volume ratios of 1:100–1:1000. Upon irradiation with laser shots and intense laser pulses (10% higher than the threshold of ionization), the analyte molecules were co-desorbed and ionized with the matrices via proton transfer reactions [4,44,45].

4.1. General characteristics of the two new matrices

Although the two new matrices (FA and MA) are insoluble in water, they can be dissolved at high concentration of organic solvents (methanol). The structures and theoretical molecular weights of the two matrices are provided in Scheme S1B.

In order to serve as an effective matrix in the MALDI-MS, the matrix must exhibit a strong absorption of laser emission (337 nm). The delocalized π(pi) electrons of the "chromophore centers" are responsible to absorb the UV laser radiation. As expected, the ionization efficiency was higher as the absorption coefficient of the matrix at the wavelength of laser (337 nm) increased. Among the various isomers of dihydroxybenzoic acids (DHB), only 2,5-DHB has been widely used as the matrix for UV-MALDI-MS [6]. However, absorption of chromophore center could result in broadening or shifting to longer wavelengths in the solid state [46]. The absorption spectra of furoic acid (FA) and mefenamic

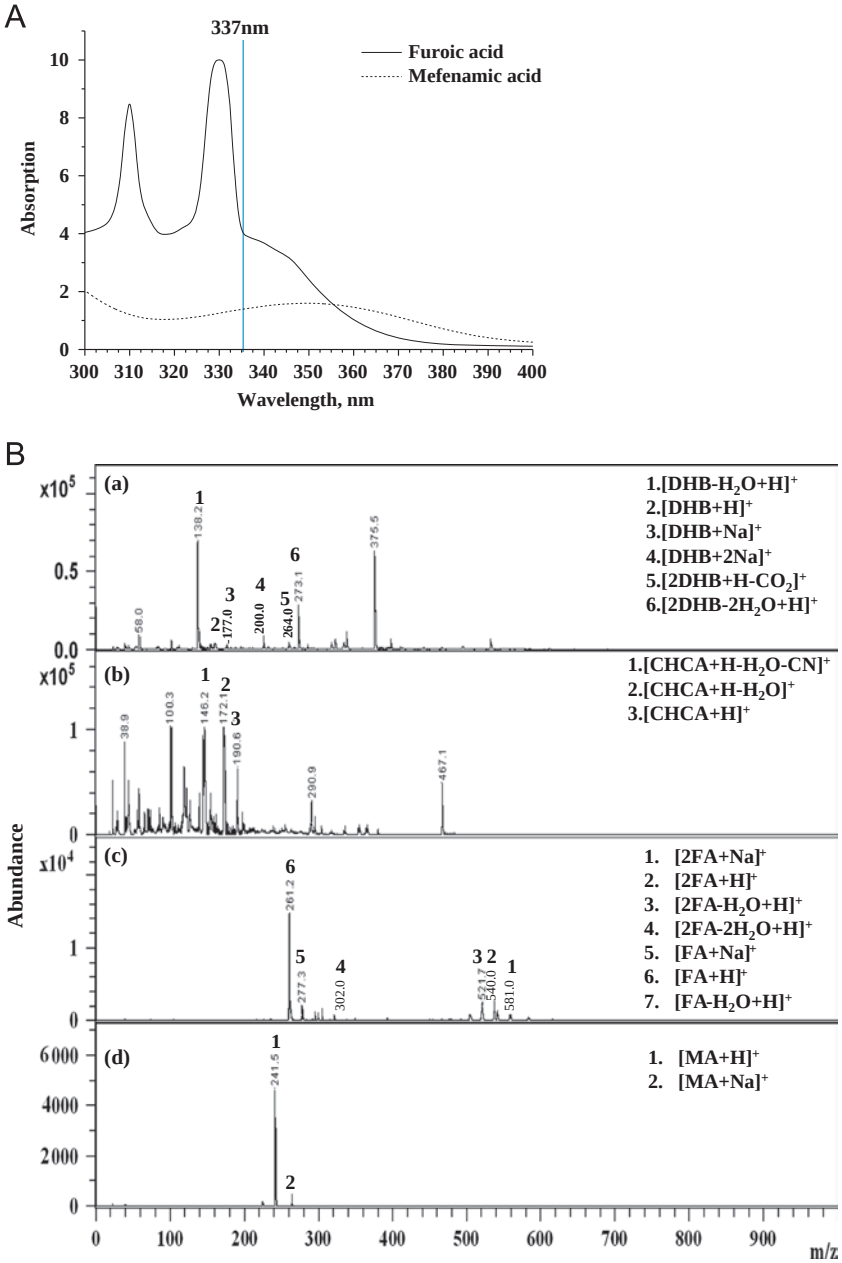

Fig. 1. (A) UV absorption spectra of furoic acid and mefenamic acid, vertical line represent the wavelength of N_2 laser (337 nm), (B) LDI-MS spectra of (a) 2,5-DHB, (b) 4-CHCA, (c) furoic acid (FA), and (d) mefenamic acid (MA), respectively.

acid (MA) are plotted in Fig. 1A. Both matrices exhibit broad absorbance around 337 nm, which reveal their potential capability to serve as matrices in the MALDI-MS.

When matrices absorb laser radiation, the evaporation of the matrix could lead to the generation of analyte ions. As the organic matrix ionizes the analytes based on proton transfer reactions, it is the reason why carboxylic acids are used as organic matrices. Furoic acid (FA) is a strong acid because of the presence of oxygen atom constrained in a five member ring as shown in Scheme S1B.

The ideal matrix should be stable under high vacuum of MALDI-MS environment. As the MALDI-MS instruments operate under high vacuum so many compounds tend to sublime. The sublimation of the organic matrix under high vacuum is the reason for MALDI-MS signal decay [47]. The 2,5-DHB easily undergoes sublimation due to low enthalpy of sublimation ($-\Delta H_{sub} = 109 \pm 3$ kJ mol^{-1}) [48]. While the mefenamic acid (MA) melts at 235.8 °C with higher enthalpy of 132.6 ± 1.8 kJ mol^{-1} [49,50]. In addition, the stability of the matrix in solution is another important concern for ideal matrix. Both conventional matrices (DHB and CHCA) have hydroxyl groups and double bonds, thus they are easy to undergo oxidation when they are stored for a period of time. So that it is recommended to use fresh matrices solution.

The interferences from the matrix molecules often lead to interpretation difficulty for mass spectra. Both nanoparticles (NPs) and organic matrices suffer from this problem [51–54]. Thus, the ideal matrix should produce none or minimum interference peaks during the MALDI-MS analysis. The 2,5-DHB produces many interferences with high intensity at *m/z* 137.1, 155.2, 177.0, 200.0, 264.0 and 273.3 Da (Fig. 1B) which were assigned as [DHB–H_2O+H]$^+$, [DHB+H]$^+$, [DHB+Na]$^+$, [DHB+2Na]$^+$, [2DHB+H–CO_2]$^+$ and [2DHB–2H_2O+H]$^+$, respectively [6]. The alpha-cyano-4-hydroxycinnamic acid (CHCA) also generates many matrix-related ions (cluster ions) at *m/z* 146.0, 172.0, 190.0, 212.0, 234.0, 335.0 and 379.0 Da which are assigned as [CHCA+H–H_2O–HCN]$^+$, [CHCA+H–H_2O]$^+$, [CHCA+H]$^+$, [CHCA+Na]$^+$, [CHCA–H+2Na]$^+$, [2CHCA+H–CO_2]$^+$, and [2CHCA+H]$^+$ ions, respectively [55]. Our new matrix furoic acid (FA) only produces intense peaks at *m/z* 279.1 and 260.0 Da which are [FA+H]$^+$ and [FA–H_2O+H]$^+$, respectively. Other

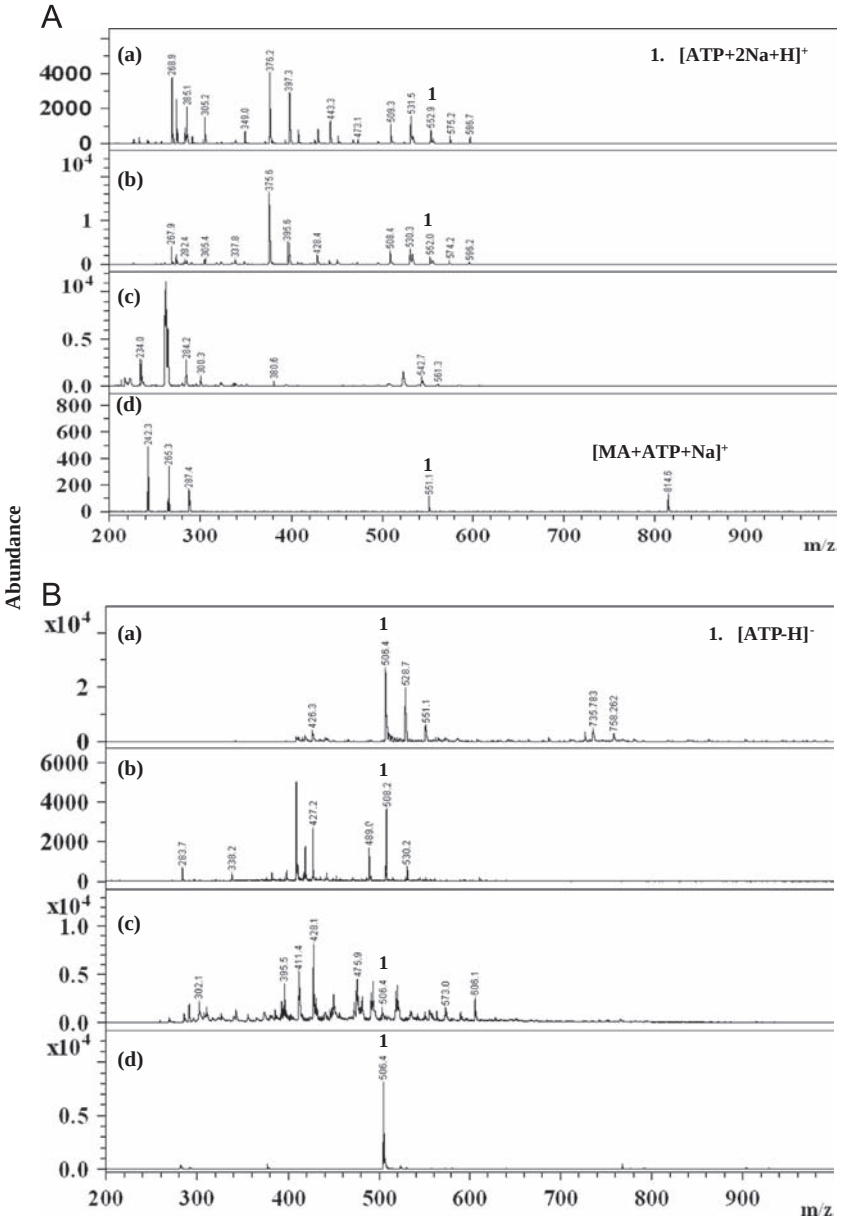

Fig. 2. MALDI-MS spectra of adenosine-5-triphosphate (ATP) in (A) positive and (B) negative modes using matrices (a) 2,5-DHB, (b) CHCA, (c) furoic acid, and (d) mefenamic acid.

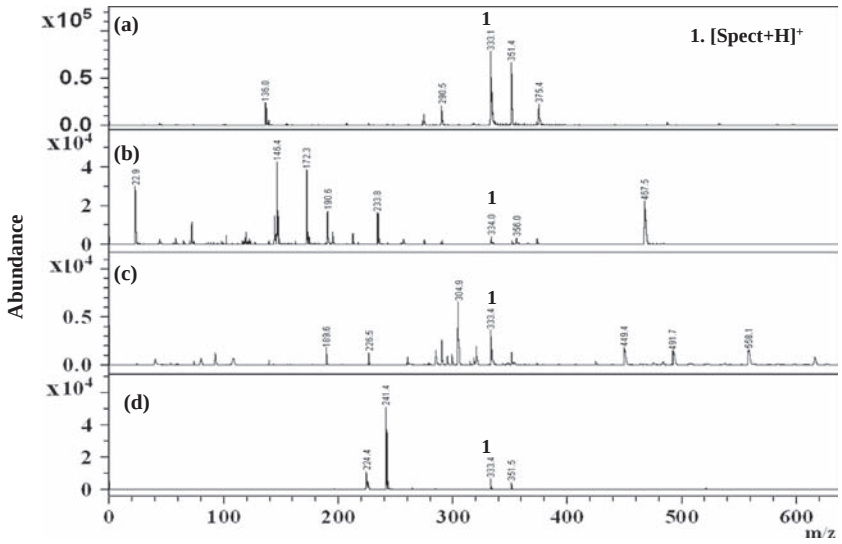

Fig. 3. MALDI-MS spectra of spectinomycin (Specr) in positive mode using matrices (a) 2,5-DHB, (b) CHCA, (c) furoic acid, and (d) mefenamic acid.

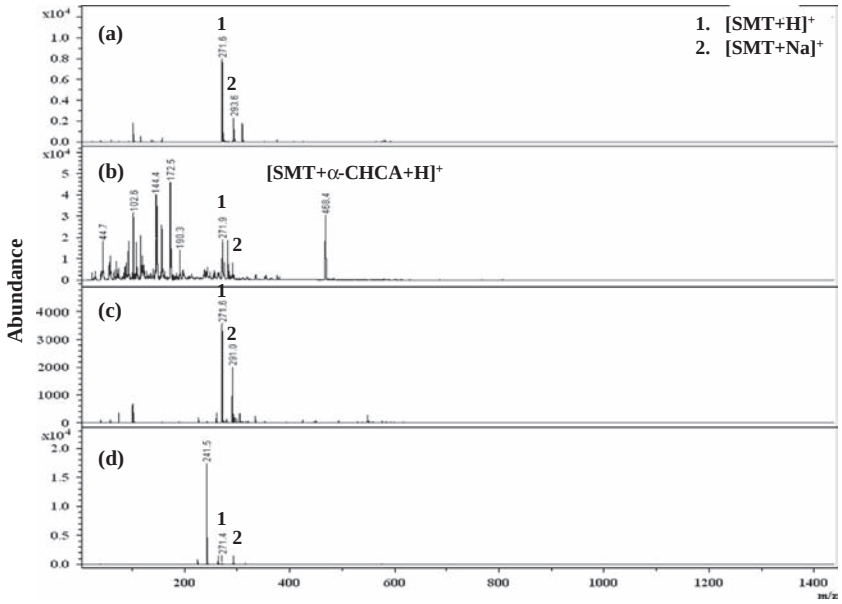

Fig. 4. MALDI-MS spectra of sulfamethazole (SMT) in positive mode using matrices (a) 2,5-DHB, (b) CHCA, (c) furoic acid, and (d) mefenamic acid.

low intensity peaks of furoic acid (FA) are reported in Fig.S2(b), Supporting information. As to the mefenamic acid (MA), it only generates two peaks at *m/z* 241.5 and 264.5 Da, which are $[MA+H]^+$ and $[MA+Na]^+$, respectively. The MA and FA are apparently superior than the DHB and CHCA to serve as MALDI matrix as they would not produce intense cluster ions or fragment ions which are easier for interpretation by MALDI-MS.

4.2. Applications of the two new matrices for a variety of compounds

To demonstrate the wide applicability of the two new matrices in the MALDI-MS, we investigated various classes of compounds including adenosine-5′-triphosphate (ATP, 0.5 μL, 10.0 nmol), spectinomycin (Spect, 0.5 μL, 14.0 nmol), glutathione (GSH, 0.5 μL, 9.0 nmol), sulfamethazole (SMT, 0.5 μL, 2.0 nmol) and gramicidin D (0.5 μL, 9.0 nmol). Their structures and molecular weights are shown in Scheme S1B. All analytes cannot be successfully ionized by direct laser desorption/ionization mass spectrometry (LDI-MS). Furoic acid (FA) and mefenamic acid (MA) can be successfully applied as new matrices for all the above analytes in both mode positive and negative mode in MALDI-MS.

Adenosine-5′-triphosphate (ATP) was obtained from the commercial source as disodium salt with molar mass at *m/z* 551.1 Da; while adenosine-5′-triphosphate molecules has a molar mass of *m/z* 507.1 Da (Scheme S1B). In the positive ion mode, both DHB and CHCA used as matrix can produce the protonated molecule ions at *m/z* 552.0 Da with extremely low abundance (Fig. 2A); while the mefenamic acid used as matrix can produce ions at *m/z* 551.1 Da ($[ATP+2Na+H]^+$) with nice resolution (Fig. 2A). The peak at *m/z* 814.6 Da ($[MA+ATP+Na]^+$) was the sodinated adduct ion of ATP with MA, (Fig. 2A). Furoic acid used as matrix cannot detect ATP in the positive ion mode due to the matrix interferences (Fig. 1B). The *m/z* 506.0 Da ($[ATP-H]^-$) can be detected using all four matrices (Fig. 2B). Among them, apparently mefenamic acid is the best matrix because it can produce the only single peak of analyte ions with high resolution and no any interference peaks.

Spectinomycin (Spect) has molar mass 332.3 Da. The four matrices produce $[Spect+H]^+$ ions at 333.0 Da (Fig. 3). In the

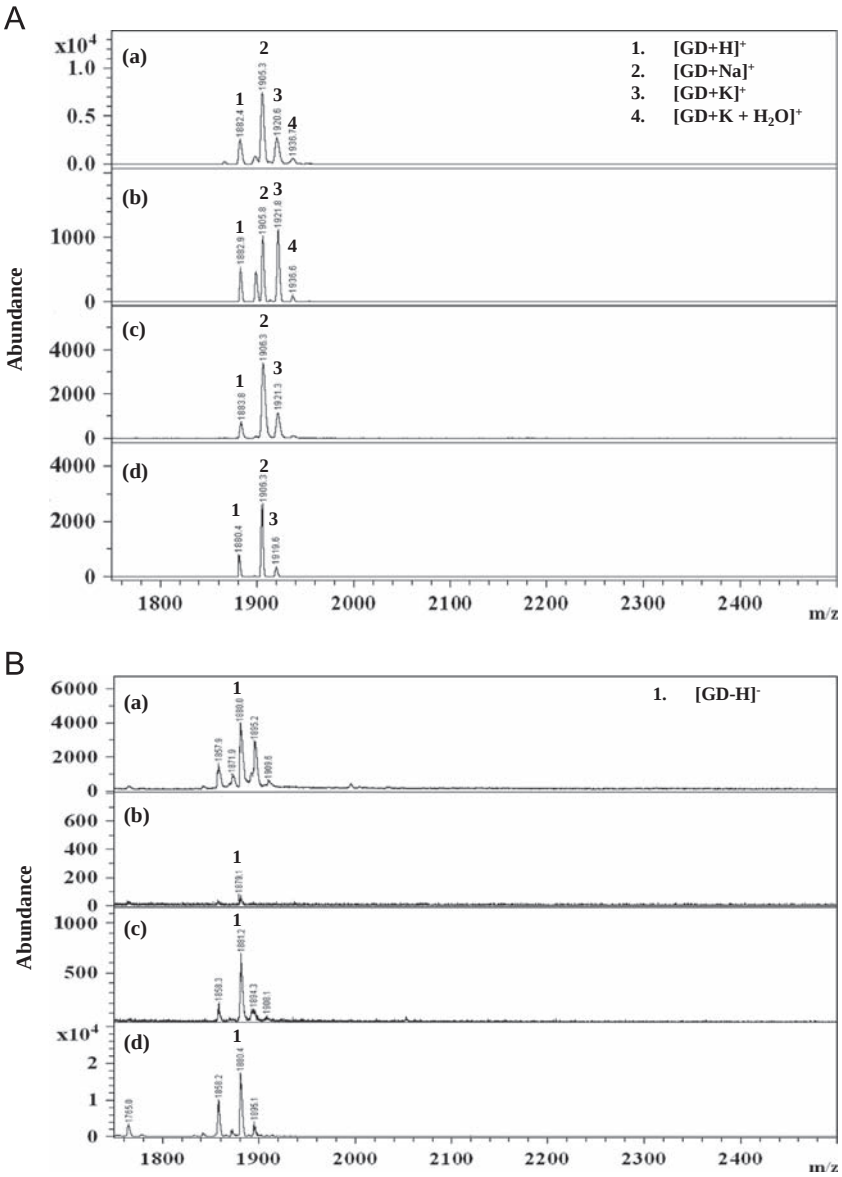

Fig. 5. MALDI-MS spectra of Gramicidin D (GD) in (A) positive and (B) negative mode using matrices (a) 2,5-DHB, (b) CHCA, (c) furoic acid, and (d) mefenamic acid.

negative ion mode, only peaks at *m/z* 331.0 Da were observed by using CHCA and furoic acid (FA) matrices (Fig. S3, Supporting information).

Sulfamethazole (SMT) produces peak at *m/z* 271.0 Da ([SMT +H]$^+$) which can be detected using all matrices (Fig. 4). This drug also produces sodium adduct ion (Fig. 4) at *m/z* 294.0 Da which was assigned as [SMT+Na]$^+$. Using CHCA as the matrix for analysis of SMT, a hetero-dimer ion at *m/z* 461.0 Da ([SMT+α-CHCA+H]$^+$) was also observed. However, mefenamic acid as matrix generated a strong peak at *m/z* 241of [MA+H]$^+$, it can ionize this molecule effectively. Both spectra obtained from MA and FA as matrices were free from interferences compared with those obtained from the conventional matrices (CHCA and DHB). SMT lacks an acidic group to achieve the ionization in the negative mode, so all matrices cannot produce any detectable signals.

Gramicidin D (GD) is a heterogeneous peptide which composes of gramicidin A, B and C, at the proportions of 80%, 6%, and 14%, respectively [18]. GD produces a protonated ion ([GD+H]$^+$) at 1882.3 Da (which is from the main component gramicidin A) using the four matrices (Fig. 5A). GD reacts with inorganic monovalent cations (such as Na^+ and K^+) can produce adduct ions at *m/z* 1905.0, 1921.0, 1936.7 Da, which were assigned as [GD+Na]$^+$, [GD +K]$^+$ and [GD+K+H_2O]$^+$, respectively. The high affinity of gramicidin toward Na^+, K^+ is the reason for gramicidin exhibit high antibiotic activity toward pathogenic bacteria. The two new matrices (MA and FA) can produce less adduct peaks compared to the conventional matrices (CHCA and 2,5-DHB) and display background free spectra. The GD antibiotic in the negative ion mode produced an ion at *m/z* 1880.0Da ([GD–H]$^-$) using the four matrices (Fig. 5B).

Glutathione (GSH) (Mol. Wt = 307.3 Da) is a tri-peptide which is composed of glycine (75.0 Da), cysteine (121.0 Da) and glutamic acid (147.0 Da). The majority of this substance was found in cells and tissue of human body in GSH form. GSH molecules are unstable due to unpaired electrons and further oxidation via the dative bond (S–S) can produce an ion at *m/z* 614.6 Da. The structures of both oxidation and reduction forms are shown in Scheme S1B. Measuring GSH/GSSG ratio is an indicator of immune dysfunctions. However, precautions such as using fresh sample and dark bottle were followed; a few matrices can show the reduced form. Fig. 6A reveals that only fuoric acid can detect it with the reduction form i.e. GSH. In the positive ion mode

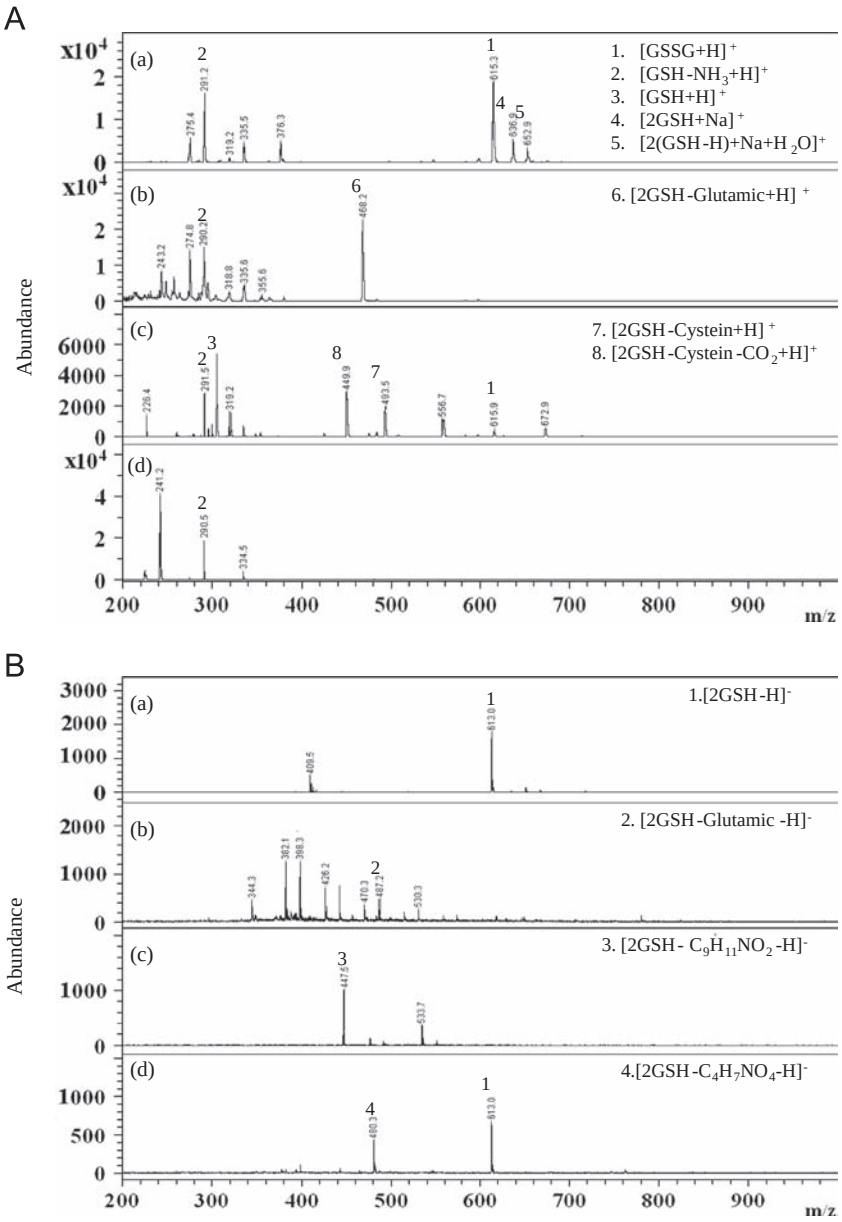

Fig. 6. MALDI-MS spectra of glutathione (GSH) in (A) positive and (B) negative mode using matrices (a) 2,5-DHB, (b) CHCA, (c) furoic acid, and (d) mefenamic acid.

Table 1
Physical parameters of the four organic matrices used in this study.

Physical parameter	2,5-DHB matrix	4HCCA matrix	Furoic acid matrix	Mefenamic matrix
Molar refractivity ± 0.3 Cm^3	36.94	49.97	78.90	72.20
Molar volume ± 3 Cm^3	98.8	133.1	217.6	200.5
Index of refraction ± 0.02	1.670	1.674	1.645	1.639
Surface tension ± 3.0 dyne/Cm	84.2	74.5	56.2	51.7
Density ± 0.06 g/Cm^3	1.559	1.420	1.283	1.203
Polarizability ± 0.5 × 10^{-24} Cm^3	14.64	19.81	32.27	28.62

(Fig. 6A), only 2,5-DHB and furoic acid can produce the dimer ion (GSSG) at *m/z* 615.0 Da ($[2GSH+H]^+$). The dimer ions observed using FA as a matrix undergoes three fragmentations indicates the unstability. Furoic acid (FA) produces a most abundant ion for GSH analysis at *m/z* 308.0 Da ($[GSH+H]^+$) which is due to the high acidity of furoic acid. All four matrices can be used to obtain a fragment ion via elimination of one ammonium molecule at *m/z* 290.0 Da ($[GSH–NH_3+H]^+$). An intense ion at *m/z* 468.0 Da ([2GSH–Glutamic+H]$^+$) using CHCA as matrix was observed. While two peaks at *m/z* 494.0 and 449.9 Da in the case of furoic acid (FA) were assigned as [2GSH–Cystein+H]$^+$ and [2GSH–Cystein–CO_2+H]$^+$, respectively. Mefenamic acid (MA) as matrix can not produce any ion related to GSSG in positive mode due to the high electronegativity of SH in glutathione and their acidic characteristic; GSSG is mainly ionized form negative ion mode. The negative ion mode was suitable for the analytes exhibit high electronegative or acidic groups. In the negative ion mode, only 2,5-DHB and mefenamic acid (MA) matrices can successfully detected the deprotonated dimmer GSH molecule at *m/z* 613.0 ($[2GSH–H]^-$) (Fig. 6B). The second ion at *m/z* 480.0 Da detected by using mefenamic acid as the matrix was the $[2GSH–C_4H_7NO_4–H]^-$ (Fig. 6B). The ion at *m/z* 447.5 Da in the case of furoic acid is assigned as $[2GSH–C_9H_{11}NO_2–H]^-$ (Fig. 6B).

Table 2
Analytical parameters of the four organic matrices for various analytes.

	Analyte	*m/z*	Matrix	FWHM[a]	*S/N*[a]	Resolution[a]
Positive mode	ATP	551	DHB	0.6	63.5	3375
			CHCA	3.1	157.6	568
			Fuoric	ND	ND	ND
			Mefenamic	0.1	170	8000
	GSH	307	DHB	ND	ND	ND
			CHCA	ND	ND	ND
			Fuoric	1	90.5	500
			Mefenamic	ND	ND	ND
	Spectinomycin	335	DHB	0.7	1685.3	448.5
			CHCA	1.3	74.4	1313
			Fuoric	1.7	90.6	201.2
			Mefenamic	0.4	257.6	893
	Sulfamethazole	271	DHB	0.6	704.1	471.5
			CHCA	16.5	31.8	19
			Fuoric	0.5	236.3	536.5
			Mefenamic	0.4	250	1000
	Gramicidin D	1882	DHB	1.4	374.8	1337
			CHCA	ND	ND	ND
			Fuoric	2	469.2	949
			Mefenamic	2.5	333.2	768
Negative mode	ATP	551	DHB	11.4	102.7	1188
			CHCA	3	14.5	364
			Fuoric	ND	ND	ND
			Mefenamic	1.4	126.8	373
	GSH	613	DHB	0.1	513.7	4326
			CHCA	ND	ND	ND
			Fuoric	0.3	85.7	1636
			Mefenamic	0.6	95.7	1055
	Gramicidin D	1880	DHB	16.6	8.4	114
			CHCA	ND	ND	ND
			Fuoric	2.8	36.7	678
			Mefenamic	24	337.4	77

[a] These data are average of 2–3 separated spectra; ND, not detected, FWHM, Full width at half maximum.

4.3. Physical characterization of the FA and MA matrices

Forming homogenous crystallization among the matrix and analytes molecules is an important factor for generating successful signals in the MALDI-MS spectra. Improvement of homogeneity is clearly dependent on the analyte and matrix properties. Several methods have been developed to improve the homogeneity of crystalline such as premixing of the analyte with the matrix before deposition on the MALDI-MS target as used herein. It has been reported that peptides and proteins tend to form large crystals using 2,5-dihydroxybenzoic acid outside the spot central [6]. Li et al. [56] used confocal fluorescence to demonstrate that the analyte is not uniformly distributed among or within the matrix crystals. So, DHB matrix was mixed with carbohydrate comatrices such as glucose, fructose, sucrose, and fucose for resolution enhancement [57]. The two new matrices (furoic acid (FA) and mefenamic acid (MA)) exhibit nice homogeneity compared to 2,5-DHB in the MALDI-MS analysis for all analytes examined in this study. This parameter i.e homogeneity is important in automated acquisition, while it is minor significant in manual acquisition in this study because we are able to direct the laser beam to the sample position where satisfactory signal intensity can be obtained. During the MALDI-MS analysis, the operator needs to carefully detect the sample spots in order to optimize ideal signals and those nice sample spots are called as “sweet spots”.

The poor reproducibility of MALDI-MS spectra obtained from using organic matrices is mainly due to two reasons: (i) low inclusion of the analyte molecules into the matrix crystal (crystallization) and (ii) variation of the analyte concentration with analyte/matrix ratio in the crystallization process. Using FA and MA as MALDI matrix can produce high reproducibility spectra for various analytes. Overlaid spectra (the repeated spectra (2-4times) were shown in one plot with different color for easy distinguish between the repeated spectrum) for mefenamic acid (Fig. S4 (A–E)) and furoic acid (Fig. S5 (A–D)) as matrix in the positive ion mode indicate stable signals with various trials. In fact, the spectra reproducibility is depend on sample preparation, impurities, sweet spots and others parameters. Even fine tune these factors can improve the sample homogenity and enhance shot-to-shot reproducibility but they cannot increase sample-to-sample reproducibility. However, we used the sample preparation/parameters of conventional matrices (DHB and CHCA); the new matrices show good reproducibility (Figs. S4 and S5). The research area of conventional matrices prior to increase homogeneity and reproducibility is still attractive. Recently, a simple method to improve and monitor the quality of CHCA matrix batches prior to sample analysis was developed to increase the reproducibility of MALDI mass spectra of peptides [58].

The physical parameters are useful to predict the strength of the interaction between the analyte with the matrix especially during crystallization. The physical parameters of all four matrices were calculated using Advanced Chemical Development Version 6 (ACD V.6, http://www.acdlabs.com/home/) and were tabulated in Table 1. The decrease in surface tension, density and index of refraction could increase the interactions between the analytes with the matrices. While increasing polarizability, molar volume and molar refractivity (Table 1) to enhance crystallization could reduce biomolecule fragmentation. Also, the two new matrices offer increase in molar refractivity than those of conventional matrices. The calculated activation energy indicates that furoic acid which has organic semiconductor properties can be activated thermally [59]. This information is able to explain why furoic acid (FA) could act as an effective matrix.

To date, DHB and CHCA are very widely used and popular organic matrices in MALDI-MS. But they exhibit several limitations such as forming a lot of interference below 500Da, unstable under vacuum due to low sublimation energy, and produce fragmentation. Intense fragmentation of analytes caused by CHCA gives an indication of the relative “heat” of a matrix, so it was called as a hot matrix. Our new matrices (FA and MA) exhibit the advantages of low cost, forming homogenous crystallization, low interferences, low fragmentation and high stability under high vacuum of MALDI-MS based on their feature of high sublimation energy.

MA and FA are superior than DHB and CHCA as they would not produce many cluster ions or fragment ions leading to easy interpretation of the mass spectra (Fig. 1B). The new matrices have significant physical improvements that make it outstanding to interact with analytes for better ionization (Table1). The two new matrices (MA ($-\Delta H_{sub} = 132.6 \pm 1.8$ kJ mol^{-1}) and FA) have high sublimation energy compared with 2,5-DHB matrix which is easily undergoes sublimation ($-\Delta H_{sub} = 109 \pm 3$ kJ mol^{-1}) [48–50]. Sublimation energy of mefenamic acid is 1.2 times higher than DHB. Although we do know the sublimation energy of fuoric acid, the sublimation energy of 2-carboxylic furan (structure close to FA) was 108.4 ± 2.2 kJ mol^{-1} which almost equal DHB value. From the structure, both new matrices (MA, FA) rich by π bonds, thus they show high UV absorption. Furthermore, they can be modified without or with little change in the matrices chromophores absorptions. Furoic acid (FA) is a strong acid because of the presence of oxygen atom constrained in a five member ring as shown in Scheme S1B. With the new matrices (MA and FA), the abundance of sodium and potassium adducts is similar to that was observed with DHB or CHCA but the protonated adducts appear to be more intense with the two new matrices.

Analytical parameters such as full width at half maximum (FWHM), signal to noise ratio (S/N), and resolution of various analytes were recorded and tabulated in Table 2. The results reveal

that mefenamic acid (MA) is a superior matrix than furoic, DHB and CHCA matrices. Data reveal improve of FWHM, *S*/*N* and resolution of some analytes. These improvements, as we alluded above, depend on the properties of the analytes. The analytical parameters reveal a promising future of the new matrices especially after optimization the parameters such as sample-matrix preparation procedures, matrix preparations, laser energy, pH, volume of deposited sample and the rates at which the sample-matrix cocrystals are grown. These parameters may be optimized in the near future.

5. Conclusion

For the first time, two new matrices, furoic acid (FA) and mefenamic acid (MA) have been proven to be effective matrices for small molecule analysis (polypeptides, proteins and drugs) in MALDI-MS. They exhibit many advantages of low cost, easy homogenous crystallization, reduced interferences, low fragmentation and high stability under high vacuum in MALDI-MS. They also have the capacity for further modification as they also exhibit new function groups compared with the traditional matrices. We believe that the two new matrices introduced in this study can contribute greatly in various fields in the near future for the studies of proteomics, biology, clinical medicine and biomedicine by MALDI-MS.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.05.050.

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