



## RESEARCH ARTICLE

# Matrix Assisted Ionization: New Aromatic and Nonaromatic Matrix Compounds Producing Multiply Charged Lipid, Peptide, and Protein Ions in the Positive and Negative Mode Observed Directly from Surfaces

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### Abstract

Matrix assisted inlet ionization (MAII) is a method in which a matrix:analyte mixture produces mass spectra nearly identical to electrospray ionization without the application of a voltage or the use of a laser as is required in laserspray ionization (LSI), a subset of MAII. In MAII, the sample is introduced by, for example, tapping particles of dried matrix:analyte into the inlet of the mass spectrometer and, therefore, permits the study of conditions pertinent to the formation of multiply charged ions without the need of absorption at a laser wavelength. Crucial for the production of highly charged ions are desolvation conditions to remove matrix molecules from charged matrix: analyte clusters. Important factors affecting desolvation include heat, vacuum, collisions with gases and surfaces, and even radio frequency fields. Other parameters affecting multiply charged ion production is sample preparation, including pH and solvent composition. Here, findings from over 100 compounds found to produce multiply charged analyte ions using MAII with the inlet tube set at 450 °C are presented. Of the compounds tested, many have –OH or –NH<sub>2</sub> functionality, but several have neither (e.g., anthracene), nor aromaticity or conjugation. Binary matrices are shown to be applicable for LSI and solvent-free sample preparation can be applied to solubility restricted compounds, and matrix compounds too volatile to allow drying from common solvents. Our findings suggest that the physical properties of the matrix such as its morphology after evaporation of the solvent, its propensity to evaporate/sublime, and its acidity are more important than its structure and functional groups.

**Key words:** Matrix assisted inlet ionization (MAII), Laserspray ionization inlet LSII), Solvent assisted inlet ionization (SAII), Laserspray ionization vacuum (LSIV), Multiply charged ions, Linear and nonaromatic matrices, Solvent-free, Dissolved MAII, Electron transfer dissociation (ETD)

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## Introduction

Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (MS) [1, 2], along with electrospray ionization (ESI) [3], has had an enormous impact on

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science. In spite of the importance and world-wide use, the ionization mechanisms of MALDI [4–8] and, to a lesser degree, ESI [9–13] are still in dispute. For MALDI, the two most prominent models are the cluster model [14, 15] and the photochemical ionization model [16, 17]. Mechanisms in MALDI have been predicated on production of singly charged ions, although Karas et al. [14] proposed a model involving multiply charged matrix:analyte clusters in which charge reduction of multiply charged ions produced from laser-induced clusters led to the observed singly charged ‘lucky survivors’. This model was subsequently modified in favor of direct production of singly charged ions from laser produced clusters [15]. More recently, this model was modified to a unified mechanism [18]. For ESI, the two prominent mechanisms are ion evaporation and charge residue [19, 20].

Laserspray ionization *inlet* (LSII) [21–25] provides an alternative approach to atmospheric pressure (AP) MALDI [26] in which production of high charge state ions, similar to ESI, using laser ablation of a matrix:analyte mixture reduces instrument-related mass range limitations and enhances structurally significantly mass selected fragmentation [23, 27]. This newly introduced inlet ionization method also offers the possibility of significantly better sensitivity than AP-MALDI because ionization occurs inside the heated inlet capillary between AP and vacuum [23, 28] eliminating so-called rim ion loss issues [29]. Besides producing ESI-like multiply charged ions, LSII was shown to involve a different ionization mechanism from MALDI [23, 28, 30]. Essentially identical results were obtained from the same bovine serum albumin (BSA) ~66 kDa sample producing up to 67 charges by introducing the matrix:analyte mixture field free into a heated inlet tube linking AP with the first vacuum region of the mass analyzer using laser ablation (LSII) or by tapping (matrix assisted inlet ionization or MAII) [31]. Thus, in LSII the laser is a high spatial resolution means of transferring the matrix:analyte to the inlet of the mass spectrometer which is important for imaging applications [27, 32–34]. Inlet ionization has recently been extended to solvents as matrices, so called solvent assisted inlet ionization (SAII), and hyphenated to liquid chromatography [35–37].

A great deal of study has gone into developing and understanding matrix compounds in MALDI including pure solids [38–57], binary solids [58–61], and liquids [62–64], but at the start of this study 2,5-dihydroxybenzoic acid (2,5-DHB) [22, 23], 2,5-dihydroxyacetophenone (2,5-DHAP) [24, 30], 2-aminobenzyl alcohol (ABA), anthranilic acid (2-aminobenzoic acid), and 2-hydroxyacetophenone (HAP) [65], were the only known MAII and LSII matrices. In LSII, matrix:analyte droplets caused by laser ablation were reported [28, 66]. 2,5-DHAP was found to require significantly lower inlet temperature than 2,5-DHB, which was attributed to lower temperature requirement for evaporation/sublimation necessary for desolvation of charged droplets (clusters) and releasing of bare analyte ions [30, 33, 66, 67]. Extending the concept of the importance of desolvation of

charged droplets in producing multiply charged ions led to the production of multiply charged ions, similar to ESI and LSII, using the matrix 2,5-DHAP with an intermediate pressure (IP) MALDI ion source; 2,5-DHB produces only low abundance multiply charged ions at IP [68]. We hypothesized that desolvation could be achieved even under vacuum conditions using more volatile matrix compounds, or alternatively, by increasing the thermal energy supplied for desolvation. By expanding the use of laserspray ionization at IP and low pressure (LP) conditions (e.g., MALDI-TOF instruments), better sensitivity might be achieved relative to AP ionization [69]. Initially, it was expected that the type and positions of functionalities in matrix compounds are important in MAII and LSI, as expected in MALDI [38–64]. In analogy to MALDI [53, 70–93], sample preparation was assumed to also be important.

Our original goal was to understand the structural elements of a matrix that are necessary for formation of multiply charged ions in LSI and MAII at AP, IP, and LP and discover more volatile matrices, which could be used under vacuum conditions. Based on assumptions of necessary structural elements for ‘good’ matrices, we looked at ion abundances and charge states produced for peptides, small proteins, and lipids using a variety of potential matrix compounds, with and without the anticipated ‘good’ structural elements. Here, we report on >100 small molecules having a wide range of structures that were tested as matrix compounds for MAII or LSII at high and low inlet temperature and for use in vacuum ionization in the absence of an inlet. Although, based on our hypothesis, it was expected that higher temperature would improve ionization through matrix desolvation, we were nevertheless astonished that at 450 °C, a large majority of the compounds tested produced multiply charged ions by MAII and, subsequently, many of these by LSII. Many of the compounds with anticipated ‘poor’ structural elements functioned as MAII or LSI matrices. Of the well performing matrices, three matrices, and binary matrix compositions thereof, produced multiply charged ions of peptides, proteins, and synthetic polymers at IP on a commercial IP-MALDI source without the need for an heated inlet. One of these compounds produced stable multiply charged ions on a commercial MALDI-TOF mass spectrometer at LP conditions [94]. Further, initial results related to analytical utility [31] and the mechanistic insights obtained from this study are addressed separately [95].

## Experimental

### Materials

Matrices were purchased from Sigma Aldrich (St. Louis, MO, USA), Fisher Scientific (Pittsburgh, PA, USA), Matrix Scientific (Columbia, SC, USA), Supelco (Bellefonte, PA, USA), and Alfa Aesar (Ward Hill, MA, USA). Acetonitrile (ACN), methanol (MeOH), trifluoroacetic acid (TFA), acetic acid (AA), formic acid (FA), and hydrochloric acid (HCl) were purchased from Fisher Scientific (Pittsburgh, PA,

USA), and angiotensin I (from human) from American Peptide (Sunnyvale, CA, USA). Insulin (from bovine pancreas), ubiquitin (from bovine erythrocytes), lysozyme (from chicken eggwhite), myoglobin, carbonic anhydrase, and ganglioside GD1<sub>a</sub> were obtained from Sigma Aldrich. Purified water was obtained from EMD Chemicals (Gibbstown, NJ, USA). Microscopy glass slides (76.2 mm×25.4 mm×1 mm) were purchased from Gold Seal Products (Portsmouth, NH, USA).

### Sample Preparation

For MAII and LSII studies, stock solutions of angiotensin I, ubiquitin, lysozyme, myoglobin, and carbonic anhydrase were prepared in pure water, ganglioside GD1<sub>a</sub> in pure methanol, and bovine insulin in 50:50 MeOH:H<sub>2</sub>O with 1 % AA to allow the sample to be completely dissolved. Matrix solutions were prepared individually by dissolving 5 mg of each matrix in 100 μL 50:50 ACN:H<sub>2</sub>O except for the following: α-cyano-4-hydroxycinnamic acid (CHCA, (*E*)-2-cyano-3-(4-hydroxyphenyl)prop-2-enoate) in 500 μL, 2,5-DHB in 50 μL, 4,4'-azobis(4-cyanovaleric acid) in 60 μL, and 2,5-DHAP in 150 μL (warmed), anthracene-9-carboxylic acid (Fisher Scientific) in 1 mL and 3,4-DHAP in 100 μL (water). For the high mass pH study, angiotensin I, ubiquitin, lysozyme, myoglobin, carbonic anhydrase and bovine insulin were diluted to 5 pmol μL<sup>-1</sup> with pure water. 2-Amino-3-nitrophenol matrix solution was pre-acidified by dissolving 10 mg in 300 μL 50:50 ACN:H<sub>2</sub>O with 1 % AA. Other acidified matrix solutions were prepared as follows: 2,2'-azobis (2-methylpropionitrile) with 1 % AA, dimethyl fumarate ([*E*]-2-butenedioic acid dimethyl ester), 2,4,6-trimethoxybenzoic acid, and 3,4,5-trimethoxybenzoic acid with 0.1 % FA. For the anthracene pH study, 5 pmol μL<sup>-1</sup> ubiquitin in water was acidified with 0.1 % FA, 0.1 % TFA, 1 % AA, and 1 % HCl, and anthracene solution in 50:50 ACN:water acidified with 1 % HCl. For matrices that were acidified to lower the pH, 0.1 % to 3 % FA was added into the matrix solution. For the solvent study, 2,5-DHAP was dissolved in 100 % ACN, 50:50 ACN:water, and acidified with 0.1 % FA, 1 % AA, 2 % AA, and 1 % HCl. For dissolved MAII and SAII studies, the stock solution of ubiquitin was diluted to 2.5 pmol μL<sup>-1</sup> with 50:50 ACN:H<sub>2</sub>O, and then 0.2 μL matrix solution was added into 200 μL of 2.5 pmol μL<sup>-1</sup> ubiquitin solution.

### Solvent-Based Sample Preparation

The layer method was used as previously described [25]. In brief, 1 μL of 5 pmol μL<sup>-1</sup> analyte solution was spotted on a glass slide and 2 μL of matrix solution was added on top (1 μL mixed and the other 1 μL without mixing), and air-dried. For azobis matrix, 1 μL of 5 pmol μL<sup>-1</sup> analyte solution was added on top of 2 μL of matrix solution spotted on a glass slide and air-dried. For binary matrices, 10 mg mL<sup>-1</sup> CHCA and 25 mg mL<sup>-1</sup> sinapinic acid (SA, 3-(4-hydroxy-3,5-dimethoxyphenyl)prop-2-enoic acid) in 50:50 ACN:H<sub>2</sub>O

were mixed with 2-nitrophenol (2-NPG, 2-nitrobenzene-1,3,5-triol) (5 mg in 100 μL) solution by 5:95 (2-NPG:CHCA, 2-NPG:SA) volume ratio for AP, 10:90 and 50:50 (2-NPG:SA) volume ratio for IP and LP measurements, respectively. Sensitivity comparison studies were obtained by spotting 1 μL of 10 and 50 fmol μL<sup>-1</sup> bovine insulin solution acidified with 1 % AA on a glass slide separately and followed by 1 μL of 2-NPG and 1 μL of 2,5-DHAP matrix solution, respectively. The dried-droplet method was used for the binary matrix study using LSI vacuum (LSIV) where 5 pmol μL<sup>-1</sup> analyte solution was premixed with the matrix solution in 1:1 volume ratio and 1 μL of the matrix:analyte mixture was spotted on a glass slide and air dried [68, 94].

### Solvent-Free Sample Preparation

Ten microliters of 1 mg mL<sup>-1</sup> (772 pmol μL<sup>-1</sup>) solution of angiotensin I in water, 10 μL of 1 mg mL<sup>-1</sup> (545 pmol μL<sup>-1</sup>) GD1<sub>a</sub> in methanol, and 10 μL of 100 pmol μL<sup>-1</sup> ubiquitin in water were dried in separate 200 μL PCR tubes in the Biodryer (BioSpec Products, Bartlesville, OK, USA), and homogenized with a TissueLyser II (Qiagen, Valencia, CA, USA), after addition of a spatula tip amount of matrices powder, similar to the published procedure [66]. The homogenization was set at 25 Hz frequency for 10 min grinding time where the ground matrix:analyte sample was simultaneously transferred to a glass slide.

### Evaporation Experiment

2-NPG, 2,5-DHAP, 2,5-DHB, CHCA, and SA matrices were used in this experiment. One μL of each matrix solution was spotted on a MALDI plate and air dried. The plate was loaded into the SYNAPT G2 MALDI source operating at IP and a picture capture of each spot was taken initially and then at 30-min intervals for 4 h and again after 14 h total. At AP, the MALDI plate with a 1 μL spot of each of the matrix solutions was placed on top of a hot plate. Observations were made over a 5-min period.

### Laser Impact Angle Experiment

2,5-DHAP, 2-NPG, and methyl succinic acid matrices were used to perform transmission geometry (TG) and reflection geometry (RG) experiments. Matrix:analyte mixture was prepared by 1:1 layer method on a glass slide and ablated using a nitrogen laser (337 nm). An optical microscope (Nikon Eclipse LV100) was used to capture photographs of the ablated area at 5× magnification similar to previous studies [28, 66].

### Inlet Ionization Mass Spectrometry

An LTQ-Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) with a commercially available heated inlet

ion transfer tube was used for MAII, LSII, SAII, and dissolved MAII studies according to the published procedures [27, 31–37, 96]. In brief, the API source housing was removed. For MAII, the sample was transferred to the inlet by tapping the dried matrix:analyte mixture applied to a glass slide against the mass spectrometer inlet [31, 96], and in LSII, the focused nitrogen laser (Spectra Physics VSL-337ND-S; Mountain View, CA, USA) ablated the solid matrix:analyte sample into the inlet [27, 31–33]. In SAII, the analyte dissolved in a solvent, and in dissolved MAII the analyte dissolved in a solvent along with dissolved matrix [34], are introduced into the mass spectrometer inlet in the liquid state using 150  $\mu\text{m}$  i.d. fused silica tubing (Polymicro Technologies, Phoenix, AZ, USA) directly [35–37], or simply pipetting the solution into the mass spectrometer inlet [37]. The temperature applied on the mass spectrometer inlet tube was 450  $^{\circ}\text{C}$  unless specified differently. Matrices that performed well at high inlet temperatures were examined as low as 50  $^{\circ}\text{C}$ . MAII and LSII mass spectra are obtained by setting the maximum injection time to 50 ms and microscans to two. Electron transfer dissociation (ETD) fragmentation was obtained on the LTQ-ETD Velos for charge state +11 of ubiquitin using dissolved MAII similar to LSII-ETD previously described [23, 27]. A Waters SYNAPT G2 mass spectrometer with IMS capability was also used for LSII employing the nanoESI source as described in detail in [24, 25]. Different designs of the homebuilt skimmer cone were used including straight copper and glass tubes and a 90 $^{\circ}$  bent stainless steel tube. The source temperature was set at 150  $^{\circ}\text{C}$ . Acquisition conditions were resolution mode with 40 V sample cone and 4 V extraction cone settings. The scan time of 1 scan per s and with up to 1 min acquisition times were used. MassLynx 4.1 software was used to extract the mass spectrum.

### *LSI Vacuum (LSIV) Mass Spectrometry*

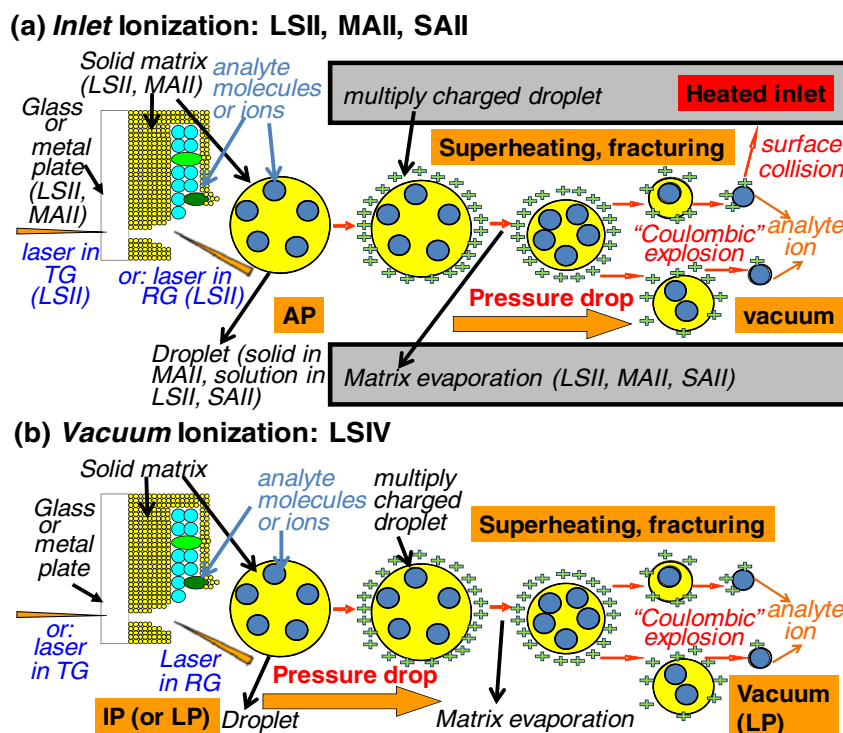
Matrices that worked well at low inlet temperature in MAII and LSII were tested for the utility to provide multiply charged ions under vacuum conditions without the benefit of a heated inlet. A variety of different binary matrix compositions were also examined using the best MAII matrix, 2-NPG, dissolved with MALDI matrices, (e.g., CHCA and SA) and air dried. A SYNAPT G2 mass spectrometer with an IP-MALDI source equipped with a Nd:YAG laser (355 nm, 200 Hz) was used, as noted in previous work [68, 94]. The instrument was operated in positive ion and sensitivity modes with 0 V on the sample plate, a 10 V “extraction,” 10 V “hexapole bias,” and 5 V “aperture 0” settings. The dried droplet method was used to spot 1  $\mu\text{L}$  of the analyte:matrix mixture onto the glass plate. After the sample plate was loaded into the MALDI source, the sample pressure was at 0.216 mbar and the drift cell pressure at 3.25 mbar. The laser fluence used was set to 170 and 225 (arbitrary units) respectively, at a 200 Hz firing rate. The total acquisition time was 1 to 2 min with 1 scan per s. Autoflex Speed mass spectrometer (Bruker, Bremen, Germany) was employed for the LP-LSI study of pure and binary matrices employing reported instrument conditions [94]. The mass

spectrometer was operated in reflectron positive ion mode. The ion source voltage (ISV) 1, ISV 2, lens voltage, rf and rf2 were set 19, 16.8, 8, 21, and 9.65 kV, respectively.

## Results

Ion abundances in the inlet ionization methods LSII, MAII, and SAII (Scheme 1, Scheme S1) have been shown to be a function of the AP to vacuum inlet temperature [23, 28, 66]. This temperature effect has been associated with matrix or solvent droplet charging and desolvation (Scheme 1). An approach for enhancing matrix dependent inlet ionization (Scheme 1a) is through discovery of matrix compounds that efficiently produce charged droplets/particles and desolvate under the available experimental conditions. Finding compounds having these features under vacuum conditions (Scheme 1b) was of interest because of the possibility of further enhancing sensitivity by eliminating losses associated with ion transfer from AP to vacuum. In order to determine structural elements useful for good matrix compounds without the necessity of absorption at a particular laser wavelength, we screened 176 compounds using MAII with an inlet temperature of 450  $^{\circ}\text{C}$  using the commercially heated inlet tube capabilities of the LTQ Velos. Of the small molecules screened, surprisingly, 137 provided abundant multiply charged ions (Scheme 2, Figure 1 and S1: common MALDI matrices and respective isomers, Scheme 3, Figure 2, and S2A: aromatic compounds, and Scheme 4, Figure 3 and S2B: nonaromatic and linear compounds). Matrices that provided the highest ion abundance at an inlet temperature of 450  $^{\circ}\text{C}$  are summarized for positive (Table S1) and negative ion mode measurements (Table S2). These matrices were subsequently analyzed at temperatures as low as 50  $^{\circ}\text{C}$  (Figure S3). Compounds that do not provide significant ions at 450  $^{\circ}\text{C}$  or increase the charge state in dissolved MAII relative to SAII are listed in Table S3.

Several matrices using solvent-free sample preparation conditions were also tested (Figure 4 and S4). The influence of the solvent composition (Figure S5), pH (Figure S6), counter anions (Figure S7), the applicability for negative mode (Figure 5 and S8), and high-mass measurements (Figure S9) were further considered for a selected set of matrix compounds. The applicability for LSI (Figure 2II.b, Figure S10) was determined for aromatic and nonaromatic compounds, and the influence of the laser alignment in TG versus RG studied (Figure S11) similar to previous reports using AP- and vacuum MALDI [30, 97, 98]. Matrices and sample preparation conditions that produced exceptional results at low inlet temperatures were further investigated on instruments lacking a heated inlet tube such as the SYNAPT G2 nano-ESI skimmer cone ion entrance, the SYNAPT G2 IP-MALDI source, and the Bruker LP-MALDI-TOF vacuum source. Binary matrices were tested at AP, IP, and LP (Figure S12). Collisions of multiply charged droplets/particles with surfaces, as well as radio frequency (rf) fields presumably aid desolvation (Figure S13). Lipids, peptides, and proteins were analyzed.



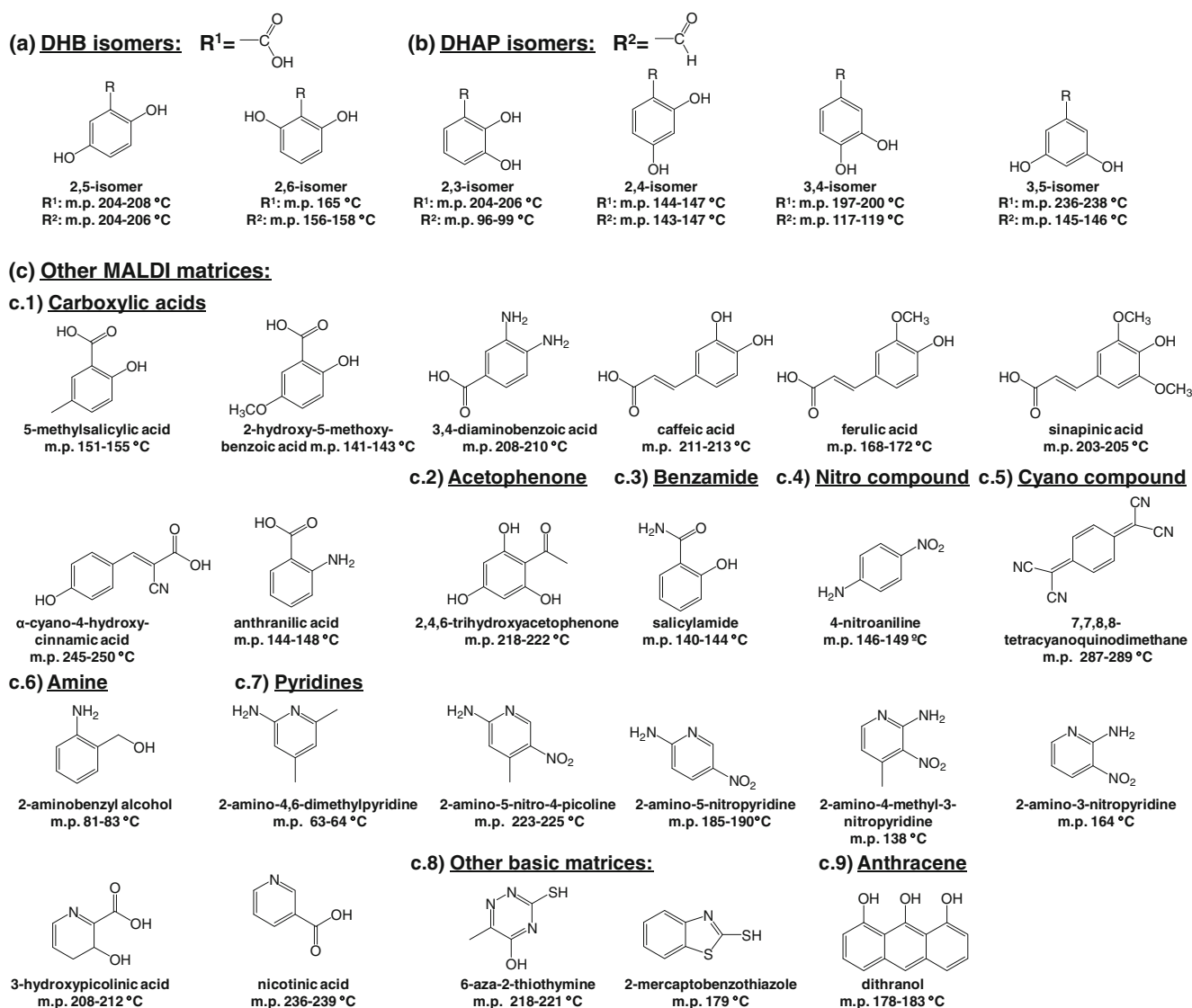
**Scheme 1.** Schematic representation of ion formation in (a) inlet and (b) vacuum ionization

### Matrices Tested using Matrix Assisted Inlet Ionization

Structural commonalities of the successful MAII matrices may reflect our selection of compounds to test based on preconceived expectations from structural elements of previously successful MALDI (Scheme 2) and LSII matrix compounds. With this caveat, the better matrix compounds were found to have two or more functional groups, one of which is a  $-OH$  or  $-NH_2$  group (Schemes 3 and 4). One of the other functional groups of the better matrices is also an electron withdrawing group consisting of  $-CO_2H$ ,  $-COCH_3$ ,  $-NO_2$ , or  $-CF_3$ , with compounds having an  $NO_2$  or other good leaving groups ('explosophores' [99]) sometimes providing exceptional performance. However, some well performing matrices were discovered that have none of these structural elements, especially with sufficient heat provided. The results relative to functional groups are summarized according to carboxylic acids (Table S1A), acetophenones (Table S1B), benzaldehydes (Table S1C), benzamides (Table S1D), phenols/alcohols (Table S1E), nitro (Table S1F), cyano (Table S1G), and halogenated compounds (Table S1H), sulfonic acids and sulfonates (Table S1I), amines (Table S1J), hydrazides (Table S1K), pyridines (Table S1L), naphthalenes (Table S1M), anthracenes (Table S1N), as well as cyclic non-aromatic (Table S1O), linear conjugated (Table S1P), linear nonconjugated (Table S1Q), and thermally labile compounds (Table S1R).

Investigations of known DHB [38, 47, 53] and DHAP [40, 41, 100] MALDI matrices, and their respective isomers all produced highly charged ions (Figure S1.A, B), with different abundances at 450 °C. Caffeic acid (3-[3,4-dihydroxyphenyl]-2-propenoic acid) [101] produces charge states for ubiquitin (MW 8560) up to +12 (Figure 1a: ubiquitin). Other known MALDI matrices [38, 42, 46, 49, 102–106] such as 6-aza-2-thiothymine (Figure 1b: myoglobin), anthranilic acid (2-aminobenzoic acid), 5-methylsalicylic acid, 2,4,6-trihydroxyacetophenone, 5-bromo-2-hydroxybenzohydrazide, 4-nitroaniline, 3-hydroxypicolinic acid, and nicotinic acid (pyridine-3-carboxylic acid) produce multiply charged MAII ions, though the abundances vary greatly (Figure S1C). The least abundant multiply charged ion formation is observed with CHCA ([*E*]-2-cyano-3-[4-hydroxyphenyl]prop-2-enoate) and SA (3-[4-hydroxy-3,5-dimethoxyphenyl]prop-2-enoic acid) matrices (Figure S12). In a temperature study from 450 to 50 °C using salicylamide (2-hydroxybenzamide) and 3,4-DHAP, the ion abundance strongly decreases with decreasing inlet temperature (Figure S3) as previously observed [23].

MAII results of hitherto unknown aromatic matrices for use in MS are shown for 1,4-dihydroxy-4,6-dimethoxybenzene (DHDMB) (Figure 2I: non-acidified) and 2-amino-3-nitrophenol (Figure 2II.a: acidified) for the analyses of lysozyme (MW 14,300) producing up to +14 charge state with +12 as the most abundant. Other well performing matrices include 2,4-dihydroxybenzaldehyde and 2-naphthol (2-hydroxynaphthalene) both producing up to +11 charge

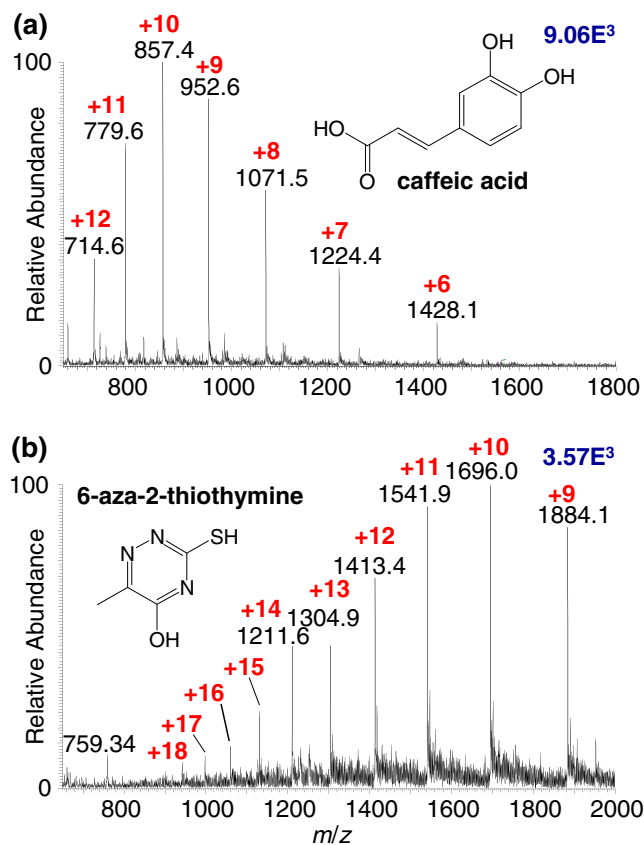


Scheme 2. Common MALDI matrices and isomers used as MAII matrices

state of ubiquitin (Figure S2A). As observed previously [31, 94–96], the chemical background of well performing MAII matrices is minute. A temperature study shows that abundant multiply charged ions are observed for acidified bovine insulin with DHDMB (Figure S3) as low as 50 °C. Resorcinol (1,3-dihydroxybenzene), one of a few MAII aromatic structures tested that diverges from 1,2-substitution did not produce multiply charged ions for ubiquitin dissolved in water, but with ubiquitin dissolved in 0.1 % FA and for insulin dissolved in 50:50 MeOH:water with 1 % AA (Figure S2, S7), abundant multiply charged ions are observed. Other aromatic matrices diverging from the 1,2-substitution, which also produce multiply charged ions of bovine insulin when acidified, include 1,3-dicyanobenzene, 1,4-dicyanobenzene, hydroquinone (1,4-dihydroxybenzene), 4-nitroaniline (p-aminonitrobenzene) and 3,5-dihydroxybenzamide (Table S1D-G). Unexpected, based on previous successful matrix compounds, the 1,3-substitution of dihydroxybenzene produces higher

charge states than 1,4-dihydroxybenzene using acidified bovine insulin (Figure S2).

Results were also obtained for linear conjugated matrix compounds carrying two functional groups (Table S1P, Figure S2B) including mesaconic acid ([*E*]-2-methyl-2-butenedioic acid) that produces up to +6 charges on acidified bovine insulin (MW 5731). However, two functional groups are not a requirement for producing highly charged ions as can be seen in case of 2,4-hexadienoic acid (Figure 3I.a). With only one carboxyl group, this matrix produces up to +12 charges on ubiquitin. A temperature study using mono-methyl fumarate ([*E*]-2-butenedioic acid hydrogen 1-methyl ester) as matrix shows that multiply charged ions of acidified bovine insulin can be observed as low as 50 °C (Figure S3). Further, succinic acid (butanedioic acid), a fully saturated matrix compound (Figure 3I.b) with two carboxylic acid groups, produces abundant, highly charged ions. Thus, neither aromaticity nor conjugation is a requirement for ion formation.



**Figure 1.** MALDI mass spectra of MALDI matrices (a) caffeic acid matrix and ubiquitin (MW 8560) and (b) 6-aza-2-thiothymine matrix and myoglobin (MW 16,952) using solvent-based sample preparation and acquired on the LTQ Velos mass spectrometer at 450 °C inlet capillary temperature

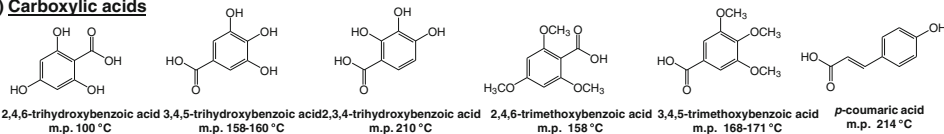
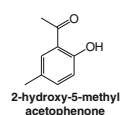
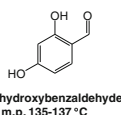
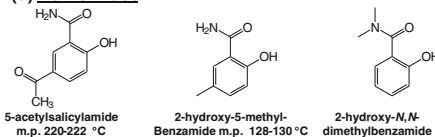
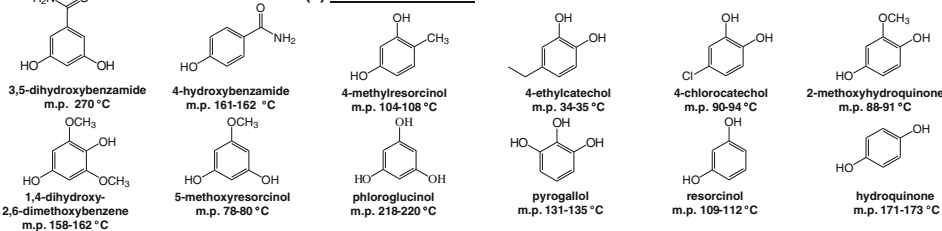
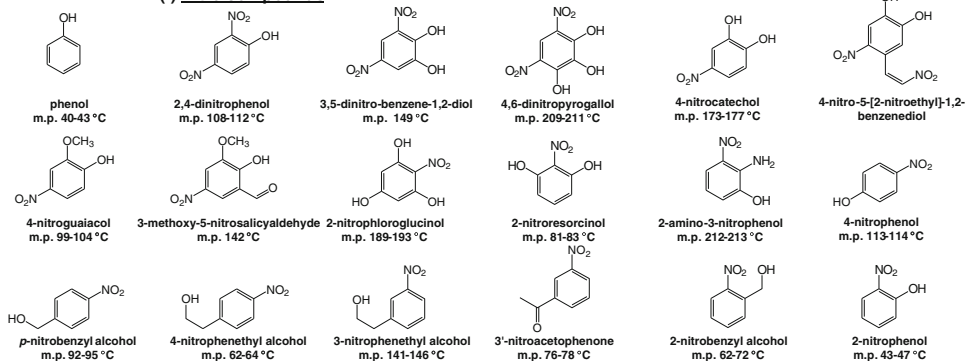
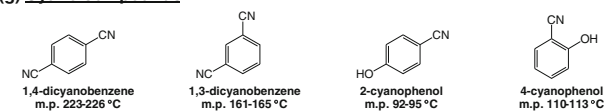
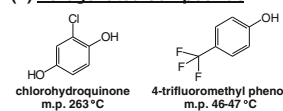
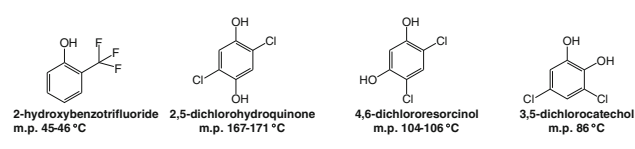
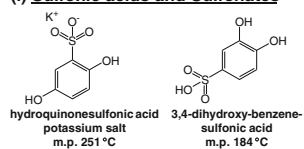
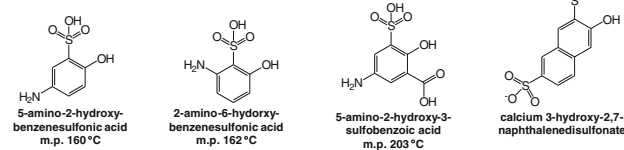
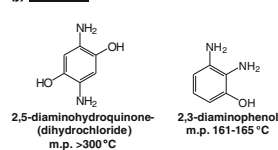
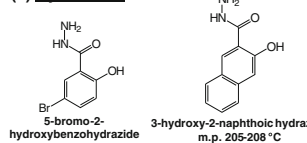
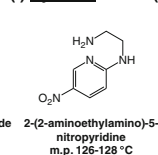
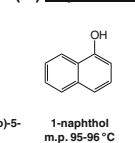
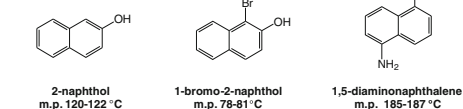
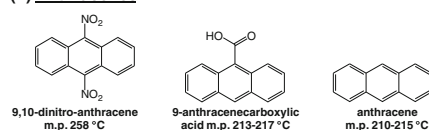
Matrix material based on thermal initiators (Scheme 4, Table S1R) used in polymer chemistry to initiate polymerization by decomposing into radicals and a good leaving group (e.g., N<sub>2</sub> in 4,4'-azobis[4-cyanovaleric acid]), provide high abundance ions with charge states up to +13 for ubiquitin (Figure 3II.a). This thermal initiator carries -COOH and -CN functional groups. The initiator 2,2'-azobis(2-methylpropionitrile) (AIBN) bears no proton donating or electron withdrawing functional groups and surprisingly produces highly charged ions from ubiquitin if acid is added to either the matrix solution (e.g., with 1 % AA) (Figure 3II.b) or the analyte (e.g., ubiquitin with 0.1 % FA or 1 % HCl) (Figure S7).

Other aromatic compounds tested (e.g., phenol and 4-trifluoromethyl phenol) as well as cyclic non-aromatic structures (e.g., 2-nitrocyclohexanol, 1,4-cyclohexane dimethanol, and 1,3-cyclohexanediol) have low melting points so that matrix evaporation is too rapid for solvent evaporation without matrix evaporation. However, solvent-free sample preparation is applicable to these samples (Figure S4 and Table S1E, H, O) because the solvent evaporation step in the presence of the volatile matrix is omitted. As is the case with the better matrices using solvent-based sample preparation, solvent-free sample preparation (e.g., 4-trifluoromethyl

phenol) provides high ion abundance and little to no chemical background. Astonishing ion abundance using 2-NPG matrix is observed with charge state +4 of angiotensin I using solvent-free sample preparation (Figure S4A); solvent-based sample preparation produces +3 as the base peak. Further, solvent-free sample preparation produces multiply charged ions with matrices having sulfonic acid or sulfonate functional groups (Table S1I) that fail to produce results using the common solvent-based sample preparation protocol developed for LSI. We attribute this to solubility restrictions in 50:50 ACN:water. For a number of matrices, using solvent-free sample preparation (Figure S4), singly charged ions are also detected, in addition to the multiply charged ions, and some basic MALDI matrices (e.g., 1,5-diaminonaphthalene, 1,8-bis(dimethylamino)naphthalene, and 9-aminoacridine) only form +1 ions (Figure S4A). This observation is in accord with previous solvent-free studies using 2,5-DHAP and 2,5-DHB [22, 66]. This study demonstrates that compounds deemed not to work as matrices may be related to how the sample was prepared and not related to structural elements.

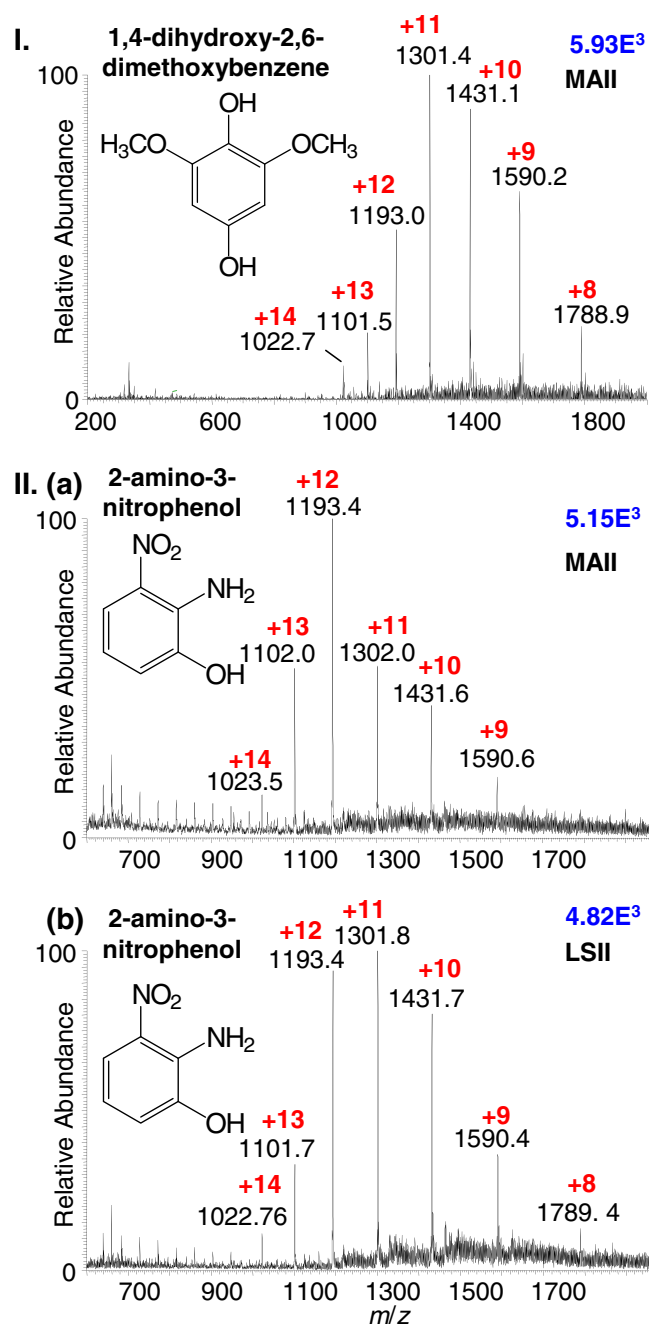
Matrices were employed that performed well applying the solvent-free sample preparation method in analyzing small proteins. Using matrix materials that have lower melting points (e.g., *cis,cis*-1,3,5-cyclohexanetriol dihydrate and 4-trifluoromethyl phenol), abundant multiply charged ubiquitin ions are observed (Figure 4) similar to solvent-based sample preparation. Interestingly, 2-NPG, a well performing matrix in solvent-based sample preparation [31, 94, 95] and as noted above for angiotensin I using the solvent-free methods, only produces highly charged ions in low abundance with ubiquitin using the solvent-free method (Figure S4A). Contrary to the better performing matrices using solvent-free analysis, the 2-NPG matrix has a relatively high melting point of 189 to 193 °C. This suggests that the matrices with lower melting points efficiently form a matrix:analyte solution in the solid state prior to the ionization and mass analyses which is in good agreement with previous solvent-free work [53, 66, 84, 90, 107–110]. The matrix 4-trifluoromethyl phenol, having a melting point of only 46 °C, was found to produce exceptional ion abundance of ubiquitin at 50 °C using solvent-free preparation (Figure 4II.b).

Gallic acid (3,4,5-trihydroxybenzoic acid), 2,4,6-trihydroxybenzoic acid, and 2,3,4-trihydroxybenzoic acid produce high charge state ions of ubiquitin (Figure S2A). However, the methoxylated derivatives, 3,4,5-trimethoxybenzoic acid and 2,4,6-trimethoxybenzoic acid, produce notably higher abundance charge states when acidified (Figure S7) but dithranol (1,8,9-trihydroxyanthracene) provides poor results when acidified. Anthracene-9-carboxylic acid and 9,10-dinitroanthracene (Figure S2A) show abundant multiply charged ions, whereas, anthracene produces multiply charged ions (Figure S7) only when acidified, demonstrating that the presence of carboxylic acid and nitro groups enhance the ionization. Ferulic acid (3-(4-hydroxy-3-

**(a) Carboxylic acids****(b) Acetophenone****(c) Benzaldehydes****(d) Benzamides****(e) Phenols/Alcohols****(f) Nitro compounds****(g) Cyano compounds****(h) Halogenated compounds****(i) Sulfonic acids and Sulfonates****(j) Amines****(k) Hydrazides****(l) Pyridine****(m) Naphthalenes****(n) Anthracenes**

Scheme 3. Novel aromatic MAII matrices





**Figure 2.** Mass spectra of lysozyme (MW 14,300) and novel aromatic matrices (I) 1,4-dihydroxy-2,6-dimethoxybenzene (DHDMB) matrix and (II) 2-amino-3-nitrophenol matrix using (a) MAII and (b) LSII applying solvent-based sample preparation and acquired on the LTQ Velos mass spectrometer at 450 °C inlet capillary temperature

methoxyphenyl)-2-propenoic acid) only forms abundant multiply charged ions of ubiquitin, up to +9, when acidified (Figure S7), but the closely related caffeic acid (Figure 1a) provides highly abundant multiply charged ions of up to +12 without acidifying. A detailed study addressing the influence of pH is included below.

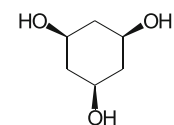
Negative mode measurements were made with a selected set of matrices (Table S2). Basic matrices such as 2-amino-

4-methyl-3-nitropyridine (Figure 5a), 2-amino-3-nitropyridine (Figure 5b), and 4-nitroaniline are applicable for negative ionization of ubiquitin and gangliosides using an inlet temperature of 450 °C with MAII (Figure S8A). One of the better performing matrices for negative mode detection of gangliosides, 4-nitroaniline, allow the use of an inlet temperature of 150 °C (Figure S8B). At this time, too few matrices have been used in the negative ion mode to suggest structural similarities of successful matrix compounds. However, matrices with functionalities that can be protonated or deprotonated (e.g., 4-nitroaniline and 2-amino-4-methyl-3-nitropyridine), operate in the positive (Figure S1C and S10) and negative mode (Figure 5, Figure S8). Basic MALDI matrices (e.g., 2-amino-3-nitropyridine and 2-amino-4-methyl-3-nitropyridine) [39], provide the ability to observe negative multiply charged MAII ions of peptides, proteins, and lipids (Figure 5, Figure S8).

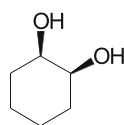
The utility of MAII matrices dissolved in, for example, ACN:water (50:50), referred to as dissolved MAII, were also tested in analogy to solid binary matrices described above and used in MALDI analyses [58–61]. The presence of 2,2'-azobis(2-methylpropane), a non-acidic matrix, shifts the charge state distribution of ubiquitin by four charges with the most abundant at +10 (Figure 6c) relative to no matrix or acid added to the solution (SAII, Figure 6b) with the most abundant charge state +6. Based on the chemical functionality, an increase in charge states, for example due to increased acidity, is not expected for this compound. Some supercharging reagents used in ESI [111–113] increase the charge states using dissolved MAII (Figure S14) (e.g., sulfolane) and other showed nearly the same charge state distribution (e.g., benzyl alcohol) relative to no matrix added (SAII). In case of 3-nitrobenzyl alcohol significant analyte-matrix adduction is observed indicating insufficient evaporation of this matrix compound at 450 °C. The use of dissolved matrices directly released from the pipette tip into the mass spectrometer inlet (Scheme S1D) is one of the easiest ways to achieve ionization without the additional steps of sample deposition and dislodging by tapping (MAII) or laser ablation (LSII/LSIV) and offers the advantages to utilize high throughput liquid handling approaches in continuous flow mode [34]. The formation of the higher charge states and ion abundance (Figure 6c) permits the use of ETD fragmentation (Figure S15).

### Matrices Tested Using Laserspray Ionization

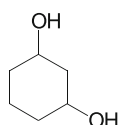
Though much can be learned from MAII studies of fundamental processes for ion formation, the use of a laser to ablate the matrix:analyte likely has broader analytical utility because of the better spatial resolution that can be achieved with laser ablation [27, 32, 33]. A number of novel MAII matrices were examined for their utility as LSII matrices. These include for example 2-amino-3-nitrophenol (Figure 2II.b), DHDMB, 2-amino-3-nitropyridine, 3,5-dihydroxybenzamide, 5-bromo-2-hydroxybenzohydrazide, 3-

**(a) Non-aromatic ring**

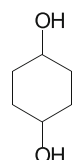
cis,cis-1,3,5-cyclohexanetriol dihydrate  
m.p. 108-111 °C



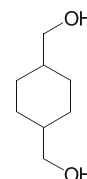
cis-1,2-cyclohexanediol  
m.p. 98-101 °C



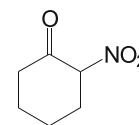
1,3-cyclohexanediol  
(cis & trans mixed)  
m.p. 30 °C



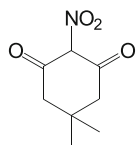
1,4-cyclohexanediol  
(cis & trans mixed)  
m.p. 98-100 °C



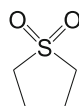
1,4-cyclohexanedimethanol  
m.p. 31.5 °C



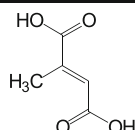
2-nitrocyclohexanone  
m.p. 39-43 °C

**(b) Linear conjugated**

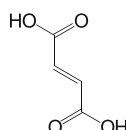
5,5-dimethyl-2-nitrocyclohexane-1,3-dione  
Liquid at room temperature



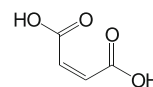
Sulfolane  
Liquid at room temperature



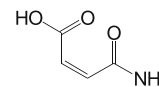
mesaconic acid  
m.p. 200-202 °C



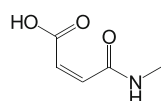
fumaric acid  
m.p. 287 °C



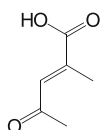
maleic acid  
m.p. 139-140 °C



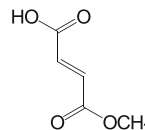
maleamic acid  
m.p. 158-161 °C



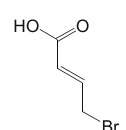
N-methylmaleamic acid  
m.p. 147-150 °C



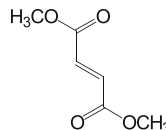
3-methyl-4-oxo-2-pentenoic acid



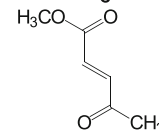
mono-methyl fumarate  
m.p. 144-145 °C



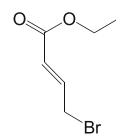
(2E)-4-bromo-2-butenoic acid  
m.p. 284 °C



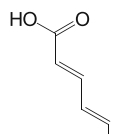
dimethyl fumarate  
m.p. 102-106 °C



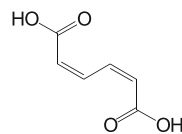
methyl 4-oxo-2-pentenoate  
m.p. 60 °C

**(c) Linear nonconjugated**

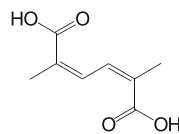
ethyl 4-bromocrotonate  
Liquid at room temperature



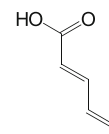
trans,trans-muconic acid  
m.p. 297 °C



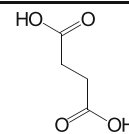
cis,cis-muconic acid  
m.p. 194-195 °C



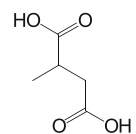
cis,cis-2,5-dimethylmuconic acid



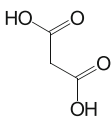
2,4-hexadienoic acid  
m.p. 132-135 °C



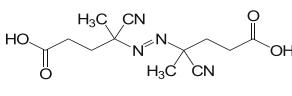
succinic acid  
m.p. 185 °C

**(d) Thermally labiles**

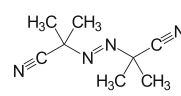
methylsuccinic acid  
m.p. 115-116 °C



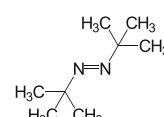
malonic acid  
m.p. 135-136 °C



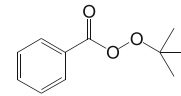
4,4'-azobis(4-cyanovaleric acid)  
m.p. 118-125 °C



2,2'-azobis(2-methylpropionitrile)  
m.p. 102-104 °C



2,2'-azobis(2-methylpropane)  
Liquid at room temperature



tert-butyl peroxybenzoate  
Liquid at room temperature

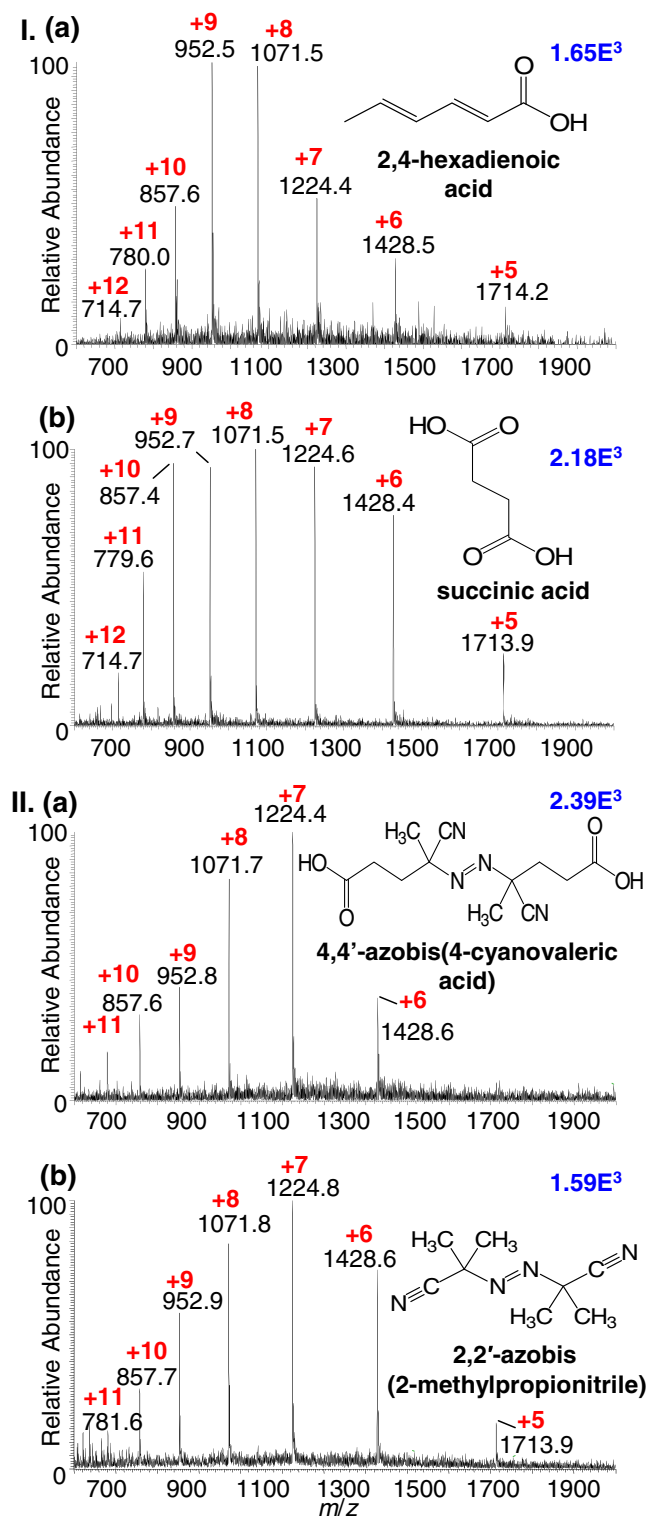
**Scheme 4.** Novel nonaromatic and linear MAII matrices

hydroxy-2-naphthoic hydrazide, 2-amino-4-methyl-3-nitropyridine, succinic acid, and methyl trans-4-oxo-2-pentenoate (Figure S6, S10). Nearly all of these compounds provide very similar results without and with use of a laser judging by the charge state distributions using the same inlet temperature. Based on previous results this is expected because the function of the laser in LSII is to ablate the matrix so that some part of the ablated material enters the inlet for subsequent ionization. Temperature studies also follow the same trend as in MAII as described above and previously observed for LSI [23, 28]. For example, the common MALDI matrix, salicylamide, produces abundant multiply charged ions of acidified bovine insulin at temperatures as low as 50 °C (Figure S10B). In some cases, signals for matrix clusters are observed and decrease with increasing inlet temperature both in MAII and LSII.

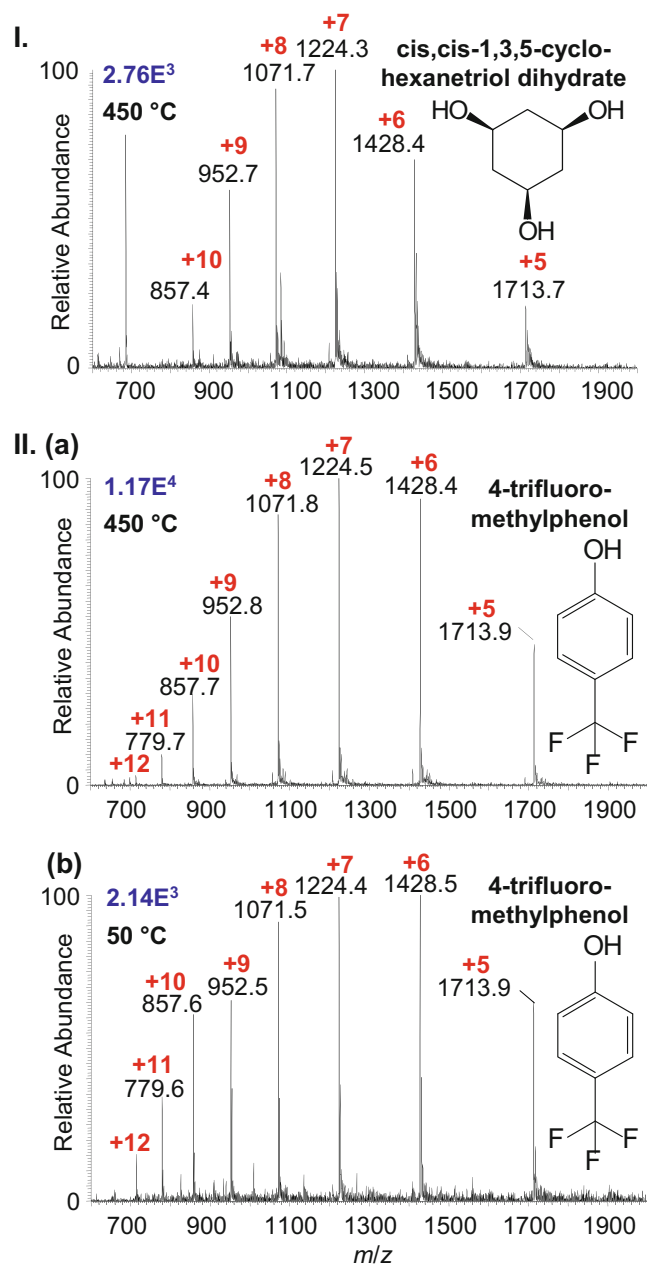
MAII matrices that work poorly, if at all, for LSII applications include liquid and sticky compounds such as

pyrogallol (1,2,3-trihydroxybenzene), resorcinol, mono-methyl fumarate, and fumaric acid (2-butenedioic acid). Some of these matrices have a notable low melting point (e.g., 2-hydroxy-5-methylacetophenone, 45 °C) so that the ablation with a laser in TG (Scheme 1a) has been difficult because of the morphology. Perhaps most surprising, LSII results for linear unsaturated compounds, such as methyl trans-4-oxo-2-pentenoate produces up to +5 charge state ions of bovine insulin and saturated compounds, such as succinic acid produces up to +6 charge state ions (Figure S10).

The result of MAII matrices that work as LSII matrices (Figure S10A) and do not bear any aromatic functionality or even conjugation was unexpected. Therefore, an additional set of experiments were performed using 2,5-DHAP, 2-NPG, and methylsuccinic acid matrices, respectively, with the laser aligned 180° (TG), as well as 0° and ~45° (RG) relative to a collection surface at the position of the mass spectrometer inlet. Microscopy of the collection surface was



**Figure 3.** MALL mass spectra of ubiquitin (MW 8560) using (I) linear matrices: (a) 2,4-hexadienoic acid, (b) succinic acid, and (II) thermal initiators: (a) 4,4'-azobis(4-cyanovaleric acid) matrix, (b) 2,2'-azobis(2-methylpropionitrile) matrix acidified with acetic acid using solvent-based sample preparation acquired on the LTQ Velos mass spectrometer at 450 °C inlet capillary temperature



**Figure 4.** MALL mass spectra of ubiquitin (MW 8560) using solvent-free sample preparation with (I) cis,cis-1,3,5-cyclohexanetriol dihydrate matrix at 450 °C and (II) temperature study with 4-trifluoromethylphenol matrix at (a) 450 and (b) 50 °C inlet capillary temperature on the LTQ Velos mass spectrometer

used to observe the results (Figure S11). Without attenuation of the laser and a single laser beam impact, complete ablation is observed where the laser beam strikes the sample 180° in TG and at 0° in RG but less clean ablation is observed with the RG 45° angle applying the same sample preparation for all three matrices in the presence of ubiquitin. The ablation diameter for 2,5-DHAP and 2-NPG is about 200 to 500 μm and for methylsuccinic acid of about 50 to 100 μm. Thus, the methylsuccinic acid results demonstrate that with sufficient

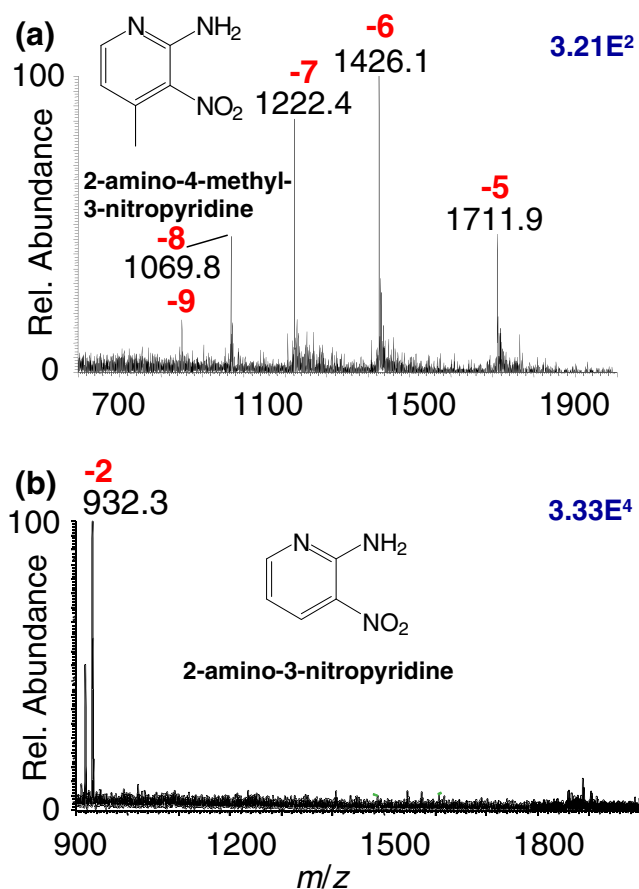


Figure 5. MALL mass spectra in negative mode measurements of (a) ubiquitin (MW 8560) and 2-amino-4-methyl-3-nitropyridine matrix, and (b) ganglioside GD1<sub>a</sub> (MW 1837 and 1866) and 2-amino-3-nitropyridine matrix using solvent-based sample preparation acquired on the LTQ Velos mass spectrometer at 450 °C inlet capillary temperature

laser energy, sample ablation can be induced without absorption at the laser wavelength.

The influence of acidic and basic conditions was studied by adding various acids and bases to either the analyte or matrix solutions using a variety of solvent compositions. It appears that matrices that are sufficiently acidic do not benefit from added acid (Figure S6). However, those matrices that do not carry acidic protons greatly benefit from acid addition (e.g., 2-amino-3-nitropyridine and anthracene) (Figure S6 and Figure 6a). A similar trend was not observed using more basic conditions by adding, for example, ammonium or sodium hydroxide.

Counter ions have been suggested to have importance in MALDI and ESI [14, 73, 114, 115] so their influence in positive (Figure S7) and negative mode measurements was also considered with LSII. The best acidifying results are obtained with 1 % HCl ≈ 0.1 % FA > 1 % AA and 0.1 % TFA. Unexpectedly, for anthracene as matrix, the highest abundance was achieved with 1 % HCl added to either the analyte or the anthracene matrix solution. This indicates that the charging of the analyte must occur in solution and the

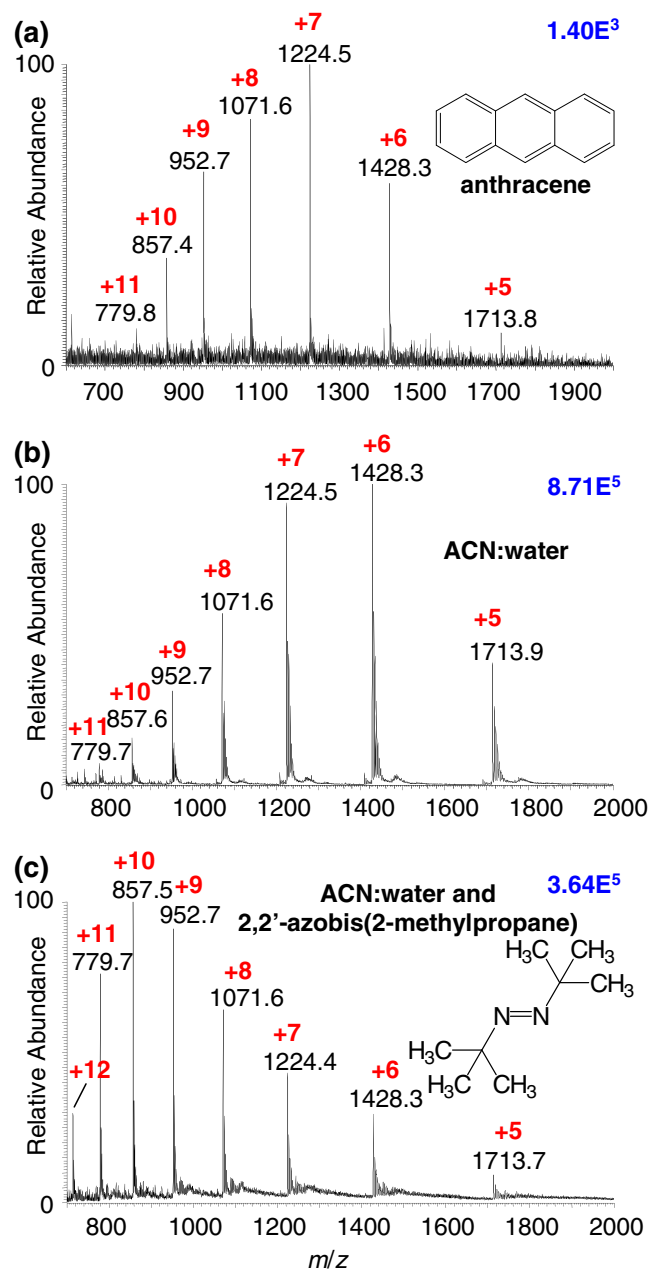


Figure 6. Mass spectra of ubiquitin (MW 8560) acquired using (a) LSII of anthracene matrix in ACN:water acidified with 1 % hydrochloric acid, (b) SALL (no matrix added to solution), and (c) dissolved MALL with 2,2'-azobis(2-methylpropane) added into the ubiquitin solution acquired on the LTQ Velos mass spectrometer at 450 °C inlet capillary temperature

matrix serves the purpose of transport into the mass spectrometer inlet. We do not see how, for example, anthracene could serve the function of counter anion. In this case, the counter anion would be the Cl<sup>-</sup> introduced when acidified. Separation of the highly charged analyte from the counter anions would likely require a means for neutralizing the anions forming gaseous compounds such as highly volatile HCl. Notable differences are also observed

with different solvent compositions used for dissolving the matrix, 2,5-DHAP, as well as the analyte (Figure S5). Water appears to be beneficial but not a requirement as good results are observed when acidifying an organic solution (e.g., ACN). More details regarding these mechanistic aspects are discussed elsewhere [95].

The importance of pH relative to the molecular weight of the analyte was studied, and using, for example, 2-amino-3-nitrophenol matrix (pH ~4.5) relative to the same matrix acidified with formic acid (pH ~2.5) shows that the pH has little influence for the analyses of peptides and small proteins such as angiotensin I, bovine insulin, ubiquitin, and lysozyme (Figure S9A–D). However, for myoglobin and carbonic anhydrase, notable enhancements are observed with the addition of acid (Figure S9E, F), which permits the use of lower inlet temperature (250 °C); without acidifying the matrix solution prior to evaporation of the solvent, the results for the larger proteins at temperatures as high as 450 °C are relatively poor. Based on these results, addition of acid for the MAII and LSII analysis of BSA, ~66 kDa, using 2-NPG as matrix, produced results with the inlet temperature as low as 200 °C [31]. We explored the use of 2,5-DHAP and 2-NPG, respectively, using the same temperature of 450 °C in the positive detection mode (Figure S16) to determine the relative sensitivity with a small protein. Using 2-NPG, 10 fmol  $\mu\text{L}^{-1}$  of bovine insulin acidified with 1% AA provides a better quality mass spectrum than 50 fmol  $\mu\text{L}^{-1}$  using 2,5-DHAP indicating an approximate fivefold increase in detection sensitivity. The 2-NPG matrix also produces highly abundant multiply charged ions at high vacuum and, therefore, we view this  $-\text{NO}_2$  containing compound as the best MAII matrix discovered in this study [31, 94, 95].

Initial success in producing multiply charged ions by inlet ionization on a mass spectrometer commercially operated with a skimmer cone ion entrance was reported using heated inlet devices and expanded to laser ablation in vacuum using matrices with low heat requirements for desolvation [24, 25, 36, 66, 94–96]. Expanding inlet ionization to mass spectrometers without a heated inlet tube is desirable. Previous studies using 2,5-DHAP [68] also demonstrated the ability to produce abundant multiply charged ions at vacuum using the SYNAPT G2 mass spectrometer's IP-MALDI source (Scheme S1E). New matrix materials found to have analytical utility for producing multiply charged ions under vacuum conditions [94] or aid in our fundamental understanding of ion formation [95] have been published. In order to determine if the volatility of a matrix and, thus, its ability to desolvate under AP and vacuum conditions, relates to the success at low inlet temperature in inlet ionization and in producing multiply charged ions in vacuum, matrix sublimation experiments were performed at IP conditions. Using methodology described in the Experimental section, the order of fastest to slowest evaporation/sublimation at AP and IP is 2-NPG > 2,5-DHAP > 2,5-DHB > CHCA and SA (Figure S17). Of these compounds, only 2-NPG and 2,5-DHAP produce abundant multiply charged ions by laser ablation at

IP and 2,5-DHB requires higher inlet temperatures at AP. This finding is in support of our working hypothesis that matrix evaporation/sublimation is important for obtaining the naked multiply charged analyte ions.

Despite exceptional performance that some of the compounds tested show as MAII and LSII matrices, the majority of these matrices did not produce ions in vacuum. Perhaps the best example is DHDMB that is substituted at the identical positions as 2-NPG and produces multiply charged ions of acidified bovine insulin even at low mass spectrometer inlet temperature of 50 °C (Figure S3) identical to 2-NPG [95]. However, the functionalities are different between these two easily desolvated MAII matrices (DHDMB: two OHs and two OMe, and 2-NPG: one  $\text{NO}_2$  and three OHs), and so are the results at vacuum. However, visual ablation was not observed for DHDMB in vacuum when lower laser power was employed, but at high laser power ablation occurred without observation of singly or multiply charged ions. Thus, ablation without strong absorption of the laser energy was insufficient for ion formation with DHDMB under vacuum conditions demonstrating insufficient matrix evaporation under vacuum conditions (Scheme 1b), despite the low temperature requirements using inlet technology (Scheme 1a) for this matrix (Figure S3). These results indicate that other parameters must have critical roles at vacuum ionization as discussed in the accompanying paper relative to the driving forces superheating and bubble formation [95], especially in the absence of an efficient heating source.

Some of the matrices evaporated/sublimed too rapidly in vacuum, a common observation in vacuum MALDI [116, 117]. For this and related reasons, the utility of binary matrices were explored. CHCA and SA matrices produce, at inlet temperatures of 450 °C, barely any ions using MAII or LSII (Figure S12A). However, with 5 % 2-NPG and 95 % CHCA abundant highly charged ions are observed. The charge state distribution is lower relative to pure 2-NPG matrix; pure 2-NPG produces lysozyme ions up to +19 (Figure S12A) and the binary matrix for SA and CHCA with 5 % 2-NPG up to +15 and +18 charges, respectively. Temperature studies of binary matrices at AP show that the inlet temperature requirements are notably lowered for SA and CHCA when 5 % 2-NPG is added. Consequently, binary matrices with 2-NPG are also useful at IP (Figure S12B) and LP (Figure S12C) producing multiply charged analyte ions similar to those obtained with 100 % 2-NPG [94]. Binary matrices extend the time before the volatile matrix component evaporates under vacuum condition, an important consideration for analyses for which longer acquisition times must be anticipated (e.g., tissue imaging [118, 119]).

## Discussion

### *Observed Trends*

Known MALDI matrices, and hitherto unknown small molecules, comprising aromatic, nonaromatic, cyclic, or

linear without conjugation, and conjugated structures with no other functionality, produce multiply charged ions using MAII with an inlet temperature of 450 °C. Initially, a trend to higher charge states and abundance with more acidic matrices was observed [67]. The new aromatic MAII matrices partially follow this trend (e.g., 2-NPG) [31], but this is not the case with, for example, DHDMB. Both structures have at least two OHs, one in ortho (DHDMB) and the other in para-substitution (2-NPG), and at least one electron withdrawing group. 2,4,6-Trihydroxyacetophenone also has the same substitution positions, but the results are not as good (Figure S1C). Similarly, phloroglucinol (1,3,5-trihydroxybenzene) and 5-methoxyresorcinol (5-methoxy-1,3-benzenediol) (Table S1E) do not produce multiply charged ions, and structures with identical substitution positions, 3,5-DHB (Figure S1A) and 3,5-DHAP (Figure S1B) produce poor results relative to other DHB and DHAP isomers. However, pyrogallol (1,2,3-trihydroxybenzene) with the same 1,3,5-substitution pattern but without the carboxylic acid or acetophenone functionality produces good results (Figure S2A). Structurally similar compounds such as 2-nitroresorcinol (2-nitro-1,3-dihydroxybenzene) (Figure S2A), 2-amino-3-nitrophenol (Figure 2II, Figure S9), other DHB isomers and DHAP isomers (Figure S1A, B) produce average to good results. However, adding an additional hydroxyl group para to the nitro group of, for example 2-nitroresorcinol, produces excellent results (2-NPG, Figure S2A) [31, 94].

Based on the ion abundance of multiply charged peptide and protein ions in the positive detection mode [94] the best performing matrix discovered in this study is 2-NPG, however, not all nitro containing compounds work well. It has been noted that the nitro functional group has properties similar to a carboxylic acid group [120, 121]. Using the structure of 2-NPG as a starting point and examining data from other useful MAII matrices one may find fuzzy commonalities between some of the better MAII matrices. For example, groups in the para position in addition to at least one further functional group, frequently electron withdrawing (e.g., 2,5-DHB, 2,5-DHAP, DHDMB, 2-NPG, and 3,5-dinitro-benzene-1,2-diol) (Figure 2, Figure S1, S2A, Table S1A, B, E, F) seem important. Perhaps a clearer trend is that the more OH groups produce higher analyte ion abundance, which is best seen with the comparison of nitro compounds with increasing numbers of -OH groups (Figure S2A); however, more heat needs to be provided for similar analyte ion abundances (Table S1F). Possibly a good leaving group is important [99].

There are exceptions to any structural commonalities observed at this point. For example, anthranilic acid performs well (Figure S1C), as does 1,3-dicyanobenzene which has no -OH groups, but produces abundant multiply charged ions in the positive mode when the analyte solution is acidified with 0.1 % FA (Figure S7). There are no acidic hydrogen atoms with this latter matrix, and so it might be assumed that analyte and residual solvent contribute the protons required for analyte ionization. Loss of volatile

components by thermal decomposition of the matrix (e.g., nitro and azo compounds) has the potential to increase ionization through rapid bubble formation [95]. This is supported by the finding that polymer initiators such as 4,4'-azobis(4-cyanovaleric acid) ( $\text{HOCCNCH}_3\text{CH-N=N-CHCNCH}_3\text{COOH}$ ) produces multiply charged analyte ions in MAII (Figure 3II.a, Table S1R); however, 2,2'-azobis(2-methylpropionitrile) ( $\text{NC(CH}_3)_2\text{C-N=N-C(CH}_3)_2\text{CN}$ ) does not, unless the matrix:analyte mixture is prepared from an acidic solution (Figure 3II.b). This may indicate that the loss of nitrogen gas from decomposition of the thermally labile matrix is important, but seems more clearly to indicate the importance of pre-charging the analyte.

The necessity for decomposition of the matrix to perform well as ionization medium must be ruled out based on the outcome of the anthracene experiments that shows excellent analyte ion abundance when acidified solutions, even with HCl, were used at inlet temperatures of 450 °C (Scheme 1a). As long as a proton source from a variety of acids is provided to the solution prior to crystallization of the matrix and analyte, good analyte ion abundances are obtained if sufficient heat is supplied. Obstruction, gases, and rf voltages can also be of a constructive means of matrix evaporation from clusters (Figure S13) and may be efficient means for mass spectrometers absent of a heated inlet.

Some of the trends noted above may be related to our initial compound selection. In attempts to expand the matrices available to LSII, several matrices initially used in MALDI, as well as DHB and DHAP isomers, were tested. Based on this predetermined set of compounds, 3,5-DHB, 3,5-DHAP, SA, CHCA, and dithranol produced poor results even at 450 °C whereas the other isomers of DHB and DHAP as well as caffeic acid worked reasonably well in producing multiply charged ions at this temperature. As with MALDI, the 2,5-isomers of DHB and DHAP worked the best in producing multiply charged ions in MAII/LSII [22]. Based on prior work, we speculated that hydroxyl groups on a benzene ring that are in the ortho- or para- positions relative to an electron withdrawing group would be a good matrix while substitution in the 3,5-position is detrimental. Interestingly, most of the compounds we expected might be good matrices produced reasonably abundant multiply charged ions at an inlet temperature of 450 °C, but so also did the compounds we thought would not be good LSII matrices.

In order to eliminate selection based on absorbance of the matrix at the laser wavelength, MAII, which does not require a laser, was used to test matrices. Upon expanding the tested compounds to those with poor absorbance at the 337 nm of a nitrogen laser, but having at least one hydroxyl group along with an electron withdrawing group, we discovered that most of these compounds provided multiply charged ions even absent an aromatic ring. Further testing demonstrated that linear or cyclic structures, even without unsaturation in the carbon structure, provided multiply charged ions of peptides and small proteins. These surprising results led to testing compounds that have no acidic protons, such as 1,4-

dicyanobenzene, 1,3-dicyanobenzene, dimethyl fumarate, anthracene, and 2,2'-azobis(2-methylpropionitrile). These matrices produce multiply charged ions if the solution from which they are dried was first made acidic.

### *Discussion Related to Observed Trends*

No structural element(s) for a MAII matrix seem essential except the need for at least one acidic proton, which, as noted above, can be circumvented by adding acid to the solution from which the matrix and analyte was dried. Based on minimum ion abundance, 137 out of 176 matrix compounds analyzed on the LTQ Velos with the inlet temperature set to 450 °C are considered MAII matrices. High abundance analyte ions are produced by 56 of these compounds. Out of these 56 better, 35 produced significant ion abundances also at 300 °C, 26 at 200 °C, and six at 50 °C. A number of compounds tested with LSII employing a nitrogen laser produced multiply charged ions even though some had weak absorption at the laser wavelength, suggesting that ablation in some cases may be a result of acoustic desorption from the glass microscope slide. Thus, known MALDI matrices and hitherto unknown small molecules, comprising aromatic, nonaromatic, cyclic, or linear carbon chains with various functional groups, with and without conjugation, and even those that are conjugated, but with no other functionality, produce multiply charged analyte ions using MAII.

The best matrices discovered so far, however, have electron withdrawing groups, but the wide range of small molecule structures that act as matrices in MAII or LSII suggest that the structure of the molecule plays a secondary role relative to physical properties such as morphology of the matrix on the target plate, its propensity to evaporate, sublime, or even thermally decompose [99], as well as its ability to solvate counter anions. A matrix that forms a layer that does not ablate well in LSII or does not produce particles when tapped against the mass spectrometer inlet in MAII, will not produce ionized analyte. Similar observations have been made in MALDI where visualization of the matrix layer is often sufficient to determine if the analysis will be successful. A 'good' matrix also strongly depends on the experimental conditions of the measurement and even the mass spectrometer used. The more limited number of compounds found suitable as matrices in MALDI may be as much a function of the restrictions imposed by the experimental conditions as any single factor studied to date.

Matrices that evaporate or sublime at lower temperature also need lower inlet temperature to produce multiply charged analyte ions suggesting that the inlet temperature may be more important for desolvation of the charged matrix droplets than for charging the matrix droplets. Matrices that are too volatile cannot be directly employed under vacuum conditions, but those that have good volatility and strong absorbance at the laser wavelength, such as 2-NPG and 2,5-DHAP might be useful as LSI matrices in IP-MALDI, and

possibly LP-MALDI-TOF mass spectrometers. Binary matrix mixtures having a volatile LSI matrix combined with a strongly absorbing MALDI matrix such as CHCA or SA also produce abundant multiply charged analyte ions somewhat reducing the problem of too rapid sublimation/evaporation of volatile matrices in vacuum.

Many matrix compounds that showed difficulty in proper crystallization, had poor solubility in the solvents used, and even those too volatile to remove solvent (e.g., water) were found to work well with peptides and proteins using solvent-free sample preparation. Thus, 52 matrices, including phenol, 2-nitrophenol, 4-trifluorophenol, and 3,4-dihydroxybenzenesulfonic acid produced high abundant multiply charged ions under solvent-free conditions. Matrices that are liquids can be easiest employed using dissolved MAII.

Considering that 137 compounds out of 176 studies serve as MAII matrices along with the observation that matrix:analyte droplets are formed during laser ablation [28, 66], we conclude that matrix compounds serve a similar purpose as solvents in SAII and ESI. Solvents such as water and methanol produce similar multiply charged ions of peptides and proteins in inlet ionization. It is, therefore, reasonable that solid matrix materials that can fulfill certain critical properties of solvents, possibly even in the molten state, will be good matrix materials provided the morphology of the dried matrix allows particles to enter the heated inlet and evaporative processes of the matrix:analyte droplets (clusters) are sufficiently fast to release the multiply charged ions. Mechanistic considerations for matrix assisted ionization, LSI, and MALDI, are provided in an accompanying paper [95].

## **Conclusion**

This work demonstrates that a wide variety of compounds are useful for matrix enhancement of ionization when suitable pH and sufficient heat or other means for desolvation of the matrix from the matrix:analyte droplets are supplied. These results when combined with similar mass spectra obtained using solvents such as water and methanol suggests that a solid matrix may serve the same purpose as a solvent in ionization. Compounds that do not have acidic protons can become useful matrices after acidifying either the analyte or matrix solution. Those matrices that have higher volatility are more likely to produce abundant highly charged ions at low inlet temperatures and some are also applicable under IP and LP conditions [31, 94, 95]. Solvent-free MAII/LSII may likely be of general value for analytes and matrices where solvent-based sample preparation is challenging, as, for example, hydrophobic and solubility restricted compounds such as lipids, hydrophobic peptides, and membrane proteins as well as matrices suffering from low solubility. Many more applications may develop through the use of binary matrices. Negative mode measurements have required prohibitive amounts of sample [65, 122], so that the initial success of a number of new matrices

providing multiply charged negative ions directly from surfaces is expected to provide opportunities for materials analyses that critically depend on negative ion formation [33, 39]. As has been reported before [94], different matrices show different propensities for different analytes (e.g., synthetic polymers) and will need to be explored in more detail in future studies. The application of use of dissolved MAII matrices may provide unknown opportunities for unadulterated tissue analyses and imaging at continuous flow [34], especially in conjunction with high performance fragmentation technologies such as ETD. Continued studies in the relationship of matrix, vacuum, thermal and laser assisted ionization for use in MS is of fundamental and practical importance [95, 123].

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