Sublimation Driven Ionization for use in Mass Spectrometry: Mechanistic Implications

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ABSTRACT: Sublimation has been known at least since the middle ages. This process is frequently taught in schools through use of phase diagrams. Astonishingly, such a well-known process appears to still harbor secrets. *Under conditions in which compound sublimation occurs, gas-phase ions are frequently detected using mass spectrometry.* This was exploited in matrix-assisted ionization in vacuum (vMAI) by adding analyte to subliming compounds used as matrices. Good vMAI matrices were those that ionize the added analyte with high sensitivity, but even matrices that fail this test often produce ions of likely matrix impurities suggesting that they may be good matrices for some compound types. We also show that binary matrices may be manipulated to provide desired properties such as fast analyses and improved sensitivity. These results imply that sublimation in some cases is more complicated than just molecules leaving a surface and that understanding the physical force responsible, and how the nonvolatile compound becomes charged, could lead to improved ionization efficiency for mass spectrometry. Here we provide insights into this process and an explanation of why this unexpected phenomenon has not previously been reported.

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Introduction

Sublimation was used by alchemist as a means of separating volatile from nonvolatile compounds[1] and in modern times is used in, for example, the electronics industry to achieve high purity.[2] Recently, an astonishingly simple process was discovered in which, during sublimation, nonvolatile analyte and/or impurities, including proteins, are transferred into the gas phase as ions.[3–5]

Small molecules known to sublime under sub-atmospheric pressure such as 3-nitrobenzonitrile (3-NBN), coumarin, 1,2-dicyanobenzene, 2-bromo-2-nitro-1,3-propanediol (or bronopol), and 2-methyl-2-nitro-1,3-propanediol, among others, when mixed in solution with analyte produce mass spectra of the ionized analyte when simply dried and exposed to conditions where the small molecule matrix sublimes.[6–8] Some subliming matrices transfer nonvolatile compounds into the gas-phase with multiple protons attached, including for example the 66 kDa bovine serum albumin protein.[4,9,10] Because sublimation is such a basic process in chemistry and used for purification purposes, understanding how these ions are produced is of fundamental importance. It is also important in mass spectrometry (MS) to have a mechanistic understanding of processes capable of transferring nonvolatile compounds into the gas phase as ions. Numerous studies over more than 30 years have attempted to determine the mechanism by which this occurs using laser ablation of a matrix compound associated with the analyte.[11] The difficulty in unraveling the mechanism of ionization in matrix-assisted laser desorption/ionization (MALDI) MS is underscored by more recent papers which argue the merits of a thermal *vs.* a photochemical initiated process.[12–14] The thermal mechanism proposes a high temperature ionization process whereas the photochemical model is based on a photoionization process. Another prominent mechanistic proposal is the cluster mechanism whereby analyte is carried into the gas phase in matrix particles/clusters having a preponderance of positive charge.[15–17] Without any knowledge of MALDI, one can conclude that either all mechanisms are partially correct, all incorrect, or a combination of correct/incorrect. In other words, 30+ years have not produced a consensus relative to the MALDI mechanism or the role of the matrix. It has been suggested that MALDI and matrix-assisted ionization (MAI) are mechanistically related.[18]

By omitting the LD in MALDI, the thermal and photochemical models are both eliminated and yet ions of small molecules as well as proteins are observed in high abundance with MAI. Because MALDI produces mostly singly charged ions, with the exception of large compounds such as proteins, and MAI produces mostly multiply charged ions even with peptides, the mechanisms of the two processes must have some key difference(s). However, the difference may lie in the energy imparted rather than the fundamental mechanistic process.[19] In other words, if Occam's razor applies, then 'the simplest solution is most likely the correct one'; MAI and MALDI would rely on the same fundamental ionization processes. This does not mean that the thermal and photoionization processes don't produce gas phase ions, but the ions they do produce may account for the background ions that are observed in MALDI, but not MAI, as well as ions of

compounds which can be vaporized without fragmentation. It can be argued that electrospray ionization (ESI),[20,21] solvent assisted ionization (SAI)[22] and all liquid based methods capable of producing gas-phase ions from nonvolatile compounds with good sensitivity also follow the same fundamental ionization process, except that the matrix is a solvent rather than a solid.[23]

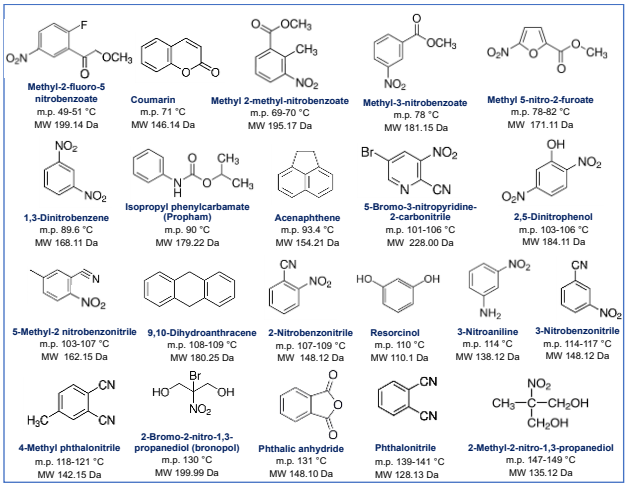
It has been proposed that the mechanism of MAI involves production of gas-phase charged matrix-analyte particles, and desolvation of these particles produce bare gas phase ions of the associated analyte. This is similar to the 'Lucky Survivor' charged particle model proposed for MALDI except that with MAI, there is no need to postulate a process for obtaining singly charged ions.[15,17] Besides the charge state difference between MAI and MALDI, the means of producing the gas phase charged particles differ. In MALDI, a laser is used to impart thermal energy into the matrix which may be responsible for the observation of low charge states. Charge stripping in ESI by addition of energy[24,25] and lower charge states in MAI with added energy[19,26] are examples supporting this hypothesis.

Here we discuss potential mechanisms whereby molecules in the solid state are transferred into the gas phase upon exposure of the sample in a suitable matrix to vacuum without application of heat, high voltage, or other energy source. The main thrust of MAI directed research has been finding matrices which produce ions from certain associated analyte with good sensitivity. It has been noted that analyte ions are detected under conditions in which sublimation is visually observable by the disappearance of the matrix.[6,27] We show here that ion production during vacuum induced sublimation is commonplace, as well as immensely useful as an ionization process for MS.[8,28] Further, manipulation of the sublimation process can be used to favorably alter the ionization process, demonstrating the importance of understanding how ions are produced from a subliming matrix.

Experimental

Chemicals and solvents, except methanol and water, were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA), including the matrices listed in **Scheme 1**. HPLC grade methanol and water were obtained from EMD Chemicals (Gibbstown, NJ, USA).

Scheme 1: Matrices studied relative to production of gas-phase ions and the relationship to sublimation. Melting points and molecular weights listed.



Analyte solutions of fexofenadine and azithromycin were prepared in ethanol, protein digest, peptides, proteins, and erythromycin were prepared in HPLC water, and diluted in HPLC water to the concentrations specified in the text. The 3-NBN matrix solution was prepared in 3:1 ACN:water at 25 mg mL^{-1}. CHCA was prepared by dissolving 2.5 mg in 100 µL ACN. The 3-NBN/CHCA matrix solution was prepared 2:1 *v*:*v* NBN:CHCA. Unless noted in the text, all other matrices were prepared as previously published.[6] Matrix solutions were used even if the matrix was not completely dissolved.

Briefly, for the probe method, matrix and analyte solutions were mixed 1:1 *v*/*v* and typically 1 µL was applied to the tip of a probe and dried before inserting into the vacuum of a mass spectrometer as previously described.[3] The time from opening the ball valve to the mass spectrometer vacuum to having the sample in the ionization region was <5 sec so that highly volatile matrices could be studied. Probe sources were used with Waters SYNAPT G2, G2S, and Thermo Q-Exactive Focus mass spectrometers.[5] Briefly, for the SYNAPT G2S, the intermediate pressure MALDI housing was modified by removing the hexapole ion guide assembly and replacing the sample introduction assembly with a flange having a ball valve to allow a probe to insert the sample to within a few mm of the entrance aperture before the step wave ion guide. No voltage was applied to the probe. For the Q-Exactive Focus, the ESI source and inlet were removed and replaced by a modified inlet with a ball valve to allow insertion of the probe either just inside the S-lens assembly or within 2 mm of the S-lens entrance. Application of +70 V to the probe for positive ion analyses was used. Different matrix solutions containing 50 fmol each of fexofenadine and angiotensin I were applied to the probe and inserted into the SYNAPT G2 as described in the text. Solutions (1 µL) of different matrices containing 5 pmol of insulin were dried on the end of the probe and inserted into the SYNAPT G2S.

Samples described in the text as acquired on an MSTM (MSTM, LLC, Hockessin, DE, USA) prototype vacuum matrix-assisted ionization (vMAI) high throughput source installed on the Q-Exactive Focus[28] were prepared by placing 0.2 to 1.0 µL of matrix:analyte solution on a glass microscope slide and dried unless otherwise specified in the text. Briefly, the glass slide can hold between 6 to 8 samples, depending on the number of channels in the spacer plate. The matrix:analyte samples align with the spacer plate channels and the sample plate and spacer plate assembly are moved between rails to sequentially expose each sample to a hole in the flange and thus to the vacuum of the mass spectrometer. A positive voltage (60-200 V) is applied to the spacer plate to direct charged particles towards the ion optics of the mass spectrometer. The charged particles are spontaneously produced when the sample is exposed to vacuum. The applied voltage is primarily

dependent on the construction of the spacer plate. The spacer plate has equally spaced 3/16" holes to allow charged particles to pass through. For positive ions, a positive voltage can be applied to a tube lens to transfer ions and charged particles exiting the hole in the flange into the S-lens. The tube for these experiments was 2" long with a 1/4" channel.

Using the MSTM vMAI source, an old solution labeled "Waters BSA Tryptic Digest, 1 pmol μL^{-1}" was mixed with different matrices and acquired as described in the text for the matrices 3-NBN and methyl 2-methyl-3-nitrobenzoate. For acquisition of the vMAI mass spectrum of lysozyme, a binary matrix having a 3:2 molar ratio of 3-NBN:CHCA in ACN (0.5 µL) was added to 0.5 µL of a 2 pmol μL^{-1} water solution of lysozyme on a glass slide. When dry, the slide was placed over a 3 mm thick spacer plate and the sample exposed to the vacuum of the mass spectrometer using positive 175 V on the spacer plate.

Results

Mechanistic considerations

Ionization by sublimation is mechanistically interesting in that it may provide insight into all processes used in MS that are capable of producing gas phase ions of nonvolatile compounds with good sensitivity. It also provides an extremely simple and sensitive ionization method that has attributes not found in other ionization approaches. The method uses small molecule matrix compounds,[6,29,30] which have in common that when exposed to sub-atmospheric pressure sublime and produce gas phase ions without external energy input.[6,29,30] That the ionization process is sublimation driven is supported by the finding that ions are observed only under conditions in which sublimation occurs, that ionization is observed until the matrix is completely sublimed or stops subliming, and that the rate of sublimation tracks with the ion abundances observed. Crushing the matrix:analyte sample mechanically under vacuum momentarily enhances the analyte ion abundance, presumably by providing a larger surface area for faster sublimation.[31] Thus, sublimation is intimately connected to the ionization event, and by inference, to the particle expulsion mechanism.

Similar to other ionization processes used in MS, it is important to know how ions are transferred into the gas phase. Direct transfer of bare ions from a solid matrix to the gas phase is unlikely on energetic grounds.[19] Latham and Stow reported that during ice evaporation, under certain conditions, emitted particles carry away charge from the surface.[32] These authors calculated that singly charged ions leaving the surface of ice as bare ions are over 50 orders of magnitude less than required for the experimentally determined charging rate, primarily because of the energetics associated with overcoming the induced charge attraction.[27,33] Because the induced field diminishes rapidly with distance, they surmised that ions can only escape the surface if they are surrounded by approximately 2,200 water molecules that provide

a distance of several nanometers between the charge and the induced charge, thus greatly reducing the force necessary for removal. Of course, there are more complexities, including the energy barrier region[34] that need to be considered to understand the release of ions, but none make the release of bare ions, especially of nonvolatile compounds, from a surface energetically favorable.[35] We can surmise emission of highly protonated bare ions are more unfavorable, thus requiring larger matrix clusters which might be a limitation to obtaining gas-phase ions of ever larger molecules from this process. However, freezing water has been photographed showing relatively large charged particles being expelled from the surface.[36] A suggested possibility is that bubbles bursting at the ice surface produces the charged gas phase particles.[37] Interestingly, excellent quality mass spectra of analytes, including small proteins, have been produced by freezing solutions of water or methanol either inside, or just outside, the mass spectrometer inlet,[6,38] thus possibly providing a commonality with matrix-assisted ionization (MAI).

One possibility for emission of charged particles from a subliming matrix is through micro pools of residual solvent in the crystallized small molecule matrices. Matrix sublimation might expose the solvent to vacuum producing an explosion of gas phase droplets possibly charged by a statistical process.[27] This is attractive as it would explain why the analyte ions observed from this process have charge states similar to those observed in electrospray ionization (ESI).[6] However, pockets of solvent within matrix crystals have not been observed with optical microscopy.[27] Additionally, a number of experiments suggest that there is insufficient residual solvent within the matrix crystals to produce charged droplets containing analyte.[27,31] Further, a statistical charging process would not be expected to provide the ion abundances observed. However, at least for production of multiply charged ions, a protic solvent, preferably water, either with the matrix or the analyte solution, is necessary for good ion abundance.[27,31]

An alternative possibility is that a sufficiently charged surface could produce a repulsive force that reduces the thermal energy necessary for ion emission, as in field desorption (FD) MS.[39–41] It might be possible for sublimation to substitute for the thermal energy applied to the FD emitter enhancing migration of analyte to areas of high field. However, there is no mechanism for producing multiply-charged ions by a pure field desorption process, nor is there a means to simultaneously produce both positive and negative ions as is observed.[42]

A remaining hypothesis is that matrix particles are the vehicles for transporting nonvolatile compounds into the gas phase. While particles have not been directly observed, possibly suggesting they are below the diffraction limit of light,

there is indirect evidence supporting this hypothesis, including difficulty in selecting ions based on their mass-to-charge, and fragmenting the gas-phase ions.[27] These results suggest that the gas-phase ions are associated with the matrix, at least in the early stages of travel to the mass analyzer, especially for the less volatile matrices.[27,31]

Observation of gas-phase analyte ions by MS also requires a charge separation process in order to produce positive or negative gas-phase ions from a neutral crystal. Because multiply-charged ions cannot be formed through gas-phase ion-molecule collisions, the charge separation process must be completed upon particle expulsion into the gas phase. Statistical charging is expected for rapid separation processes where charge equilibrium is a slower step.[19] However, statistical charging of submicron sized particles is expected to impart too few charges to account for the ion abundance or multiple charges observed with vMAI.[43] A thermal gradient between the center and surface of a particle can result in excess charge to surfaces, and preferentially removing the surface layers of ice crystals has been proposed as a mechanism for nonstatistical charge separation in thunderstorms.[19,32,33] Another attractive alternative is the mechanism for charge separation which occurs during crystal fracturing and leads to triboluminescence, also termed fractoluminescence.[44,45] In this case, the radiation produced upon crystal fracturing is believed to be caused by a discharge between oppositely charged fractured surfaces. These charges, in the absence of a discharge, will reside on the particles expelled into the gas phase. Pockets of solvent in the crystal could potentially produce an electrospray emission of charged solution droplets due to the field produced by these charges. The better performing matrices have been shown to generate more charge separation than poor performing ones.[27] Thus, two requirements for a useful vMAI matrix are the ability to sublime under the conditions of the experiment and the need for an efficient charge separation mechanism.

The vMAI matrices 3-NBN, coumarin, propham, acenaphthene, resorcinol, 1,3-dinitrobenzene, and phthalic anhydride are known to triboluminescence during crystal fracturing.[46] The overrepresentation of vMAI matrices with triboluminescence properties may, to some extent, be because such compounds were tested because of their good charge separation properties.[45] The same may also be true for nitro or cyano groups, which because the first vMAI matrix discovered, 3-NBN, had both, and thus such compounds were initially selected for study. Nevertheless, the percentage of compounds known to triboluminescence which are vMAI matrices is high relative to the percentage of the total number of compounds studied. Three of the best vMAI matrices, 3-NBN, coumarin and propham perform exceptionally well with our standard mixture of drugs and peptides based on ion abundance and limited background ions. Just as with compounds known to

triboluminescence, there are no chemical features that set vMAI matrices apart. It remains to be seen if, like sublimation, triboluminescence is a requirement for producing ions in vMAI.

In mass spectrometry, the focus is on producing ions, but experiments have shown that relative to sublimation, ion emission is a minor process with vMAI matrix compounds. This was demonstrated by placing a nonvolatile peptide on the vMAI probe with a 3-NBN matrix solution, dried, inserted into the ionization region, and allowed to completely sublime while acquiring mass spectra. Little change in peptide ion abundance is observed after repeated additions of only matrix solution to the probe followed by acquiring mass spectra until complete sublimation.[3] Thus, even though this sublimation driven ionization process has similar sensitivity to MALDI, it is also inefficient, not unlike MALDI, leaving most of the nonvolatile material on the probe to be removed from the instrument as would be expected for a purely sublimation driven process.[45]

In order to get an estimate of the ion to neutral ratio, a rough comparison was made with the Waters SYNAPT G2 using vMAI against MALDI measurements previously made on a homebuilt time-of-flight mass spectrometer. The results, described in the supplemental, show the ion-to-neutral ratio for the 3-NBN matrix to be between $e^{-13} – e^{-12}$, and for the analyte bradykinin between $e^{-8} – e^{-7}$. Because it took ca. 2 minutes to load the vMAI sample into the ionization region using the SYNAPT G2, much of the matrix had sublimed so that these values are low and thus provide only ballpark numbers. Nevertheless, they provide a rationale why sublimation can be used as a purification process. The high ion transmission efficiency of vacuum ionization compensates for the lower ionization efficiency of vMAI and vMALDI relative to ESI. The presumed lower ionization efficiency of vMAI relative to MALDI and yet producing comparable sensitivity may be related to the continuous ionization process in vMAI *vs.* discreet ionization events in MALDI.

Examples of Sublimation Driven Ionization

MH^+ ions of vMAI matrices are usually observed along with other matrix related ions such as, for example with NBN, a protonated oxidation product (*m*/*z* 166) and NBN trimer (*m*/*z* 445). Other ions of unknown origin are also observed which are believed to be associated with impurities in the matrix. This is supported by the same matrix compound from different manufacturers producing different background ions. Because ions are observed from nonvolatile analyte compounds added to the matrix, any process requiring vaporization before ionization is ruled out as a source of gas phase ions. An interesting and potentially important finding is that some matrices which fail to produce ions from our standard drug/peptide mixture, nevertheless produce ions of unknown origin. A knowledge of the structure

of these unknown compounds may uncover matrices which broaden the compound classes which are efficiently ionized by vMAI.

An example of acquiring mass spectra using the standard drug/peptide mixture is shown by the following. By adding low concentration solutions of known analytes to a matrix solution and drying before insertion into the vacuum of the mass spectrometer, without use of an energy source for ionization, the relative efficiency of ionization by each matrix for the compounds studied can be determined. The results, similar to MALDI, show that the process for producing gas-phase ions from the added analyte is selective and matrix dependent. For some matrices, the added analyte produces no observable analyte ions as demonstrated in **Figure 1A** for the matrix acenaphthene with the added analytes being 50 fmol each of the peptide angiotensin I and the drug fexofenadine using a probe device for sample insertion into vacuum.[3]

The molecular weight of acenaphthene is 154.12 so that the ion observed at mass-to-charge (*m*/*z*) 154.14 is presumed to be the molecular radical cation of the matrix, and the ion at *m*/*z* 153.13 is from loss of a hydrogen radical. Other ions in this mass spectrum are unidentified. An interesting observation is that phenothiazine as analyte is ionized by vMAI, so far only with acenaphthene as matrix, and then only as a radical cation (Supplemental **Fig S3**). This demonstrates that the determination of a good matrix must be qualified with the analytes studied.

Phthalic anhydride as a matrix provides an intermediate case in which low abundant ions of

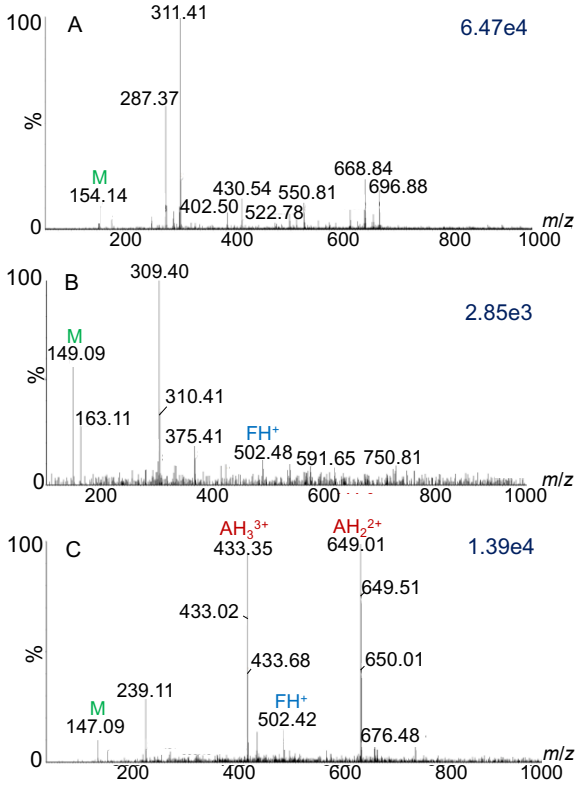


Figure 1: Mass spectra produced during sublimation of **A**) acenaphthene, **B**) phthalic anhydride, and **C**) coumarin as matrices (M) all containing angiotensin I (A) and fexofenadine (F) in solution that is applied to a probe tip, dried, and inserted into the vacuum ionization region of a Waters SYNAPT G2 mass spectrometer. The amount of each analyte applied to the probe was 50 fmol.

the added analyte are observed at *m*/*z* 502.49 (singly protonated fexofenadine) (**Fig. 1B**). The protonated molecular ions of the matrix produce a signal at *m*/*z* 149.10, but other ions are unidentified with ions at 283.35 and 311. 41 having moderate abundances. On the other hand, coumarin as matrix with the same analytes produces abundant ions of the analyte including the doubly (*m*/*z* 649.51) and triply protonated angiotensin I, and singly

charged ions of fexofenadine (**Fig. 1C**). Despite the low amount of analyte used in this study, the background ions, including the protonated matrix ion at *m*/*z* 147.10, are in low abundance relative to the analyte ions. Because all of these matrices produce ions, a charge separation process does occur under the conditions of the experiment. Even though the charge separation process is clearly more efficient with some matrices than others, the difference in relative ionization efficiency for the different analytes must be due to factors not related to the charge separation process.

Just as with the exemplified matrices discussed above, all of the compounds we have studied (**Scheme 1**) which sublime when placed in vacuum, also produce gas phase ions, although not always with the added analyte, and with different efficiency and ion current profile. While conditions may not be found in which every compound which sublimes produces gas phase ions, it is clearly a common process and one that has not previously been associated with the sublimation process. The different results obtained using different matrices are illustrated using bovine insulin in which a 10 pmol water solution mixed 1:1 with a matrix solution was applied to a the probe, dried, and inserted into the vacuum of the mass spectrometer.[3] The data acquired with coumarin as the matrix is shown in **Figure 2A & 2B**. The total ion abundance (TIC) rises to 1e^6 and drops quickly so that most of the ion abundance occurs within 2 minutes. The mass spectrum shows only insulin ions with charge states ranging from +3 to +6. On the other hand, using the same analyte but with bronopol as the matrix, no ion current is observed

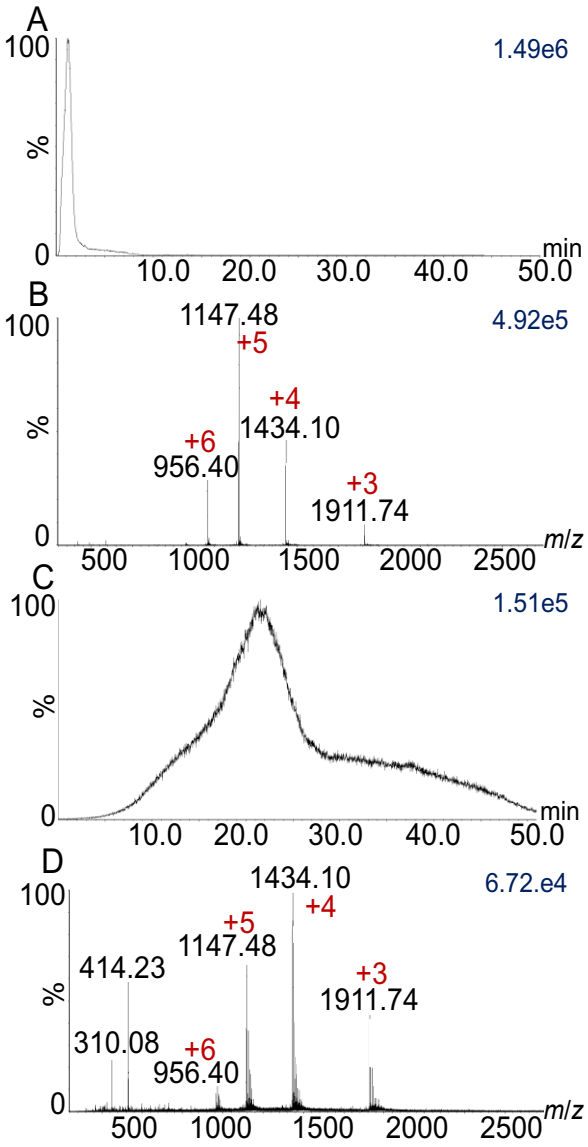


Figure 2: (**A** and **C**) Total ion current (TIC) and (**B** and **D**) mass spectra of bovine insulin using coumarin and bronopol, respectively as the vMAI matrices. The samples were introduced to the vacuum of a Waters SYNAPT G2S mass spectrometer using probe introduction. Ionization tracts the rate of sublimation for each matrix.

for the first 5 min and then the TIC slowly increases until it reaches about 1e^5 after 22 min and slowly decays over the next 30 min (**Fig. 2C & D**). The differences in the TIC between bronopol and

coumarin as matrices is directly related to the rate of sublimation of the respective matrices which is likely due to the hydroxy groups and bromine on bronopol reducing sublimation relative to coumarin. The multiply charged ions from insulin are the most abundant ions in the bronopol vMAI mass spectrum, but background ions are observed with significant abundance, and unlike the other matrices, bronopol produces adducts of the multiply charged insulin ions. Using the matrix 3-nitroaniline provides yet another TIC profile which begins to show ions after about 30 second, increases to a maximum after 8 min with ion abundance of ca. $1e^4$ and then slowly decreases over the next *ca.* 12 min (**Fig. 3**). Ions of insulin are observed, but in low abundance which are best observed in the

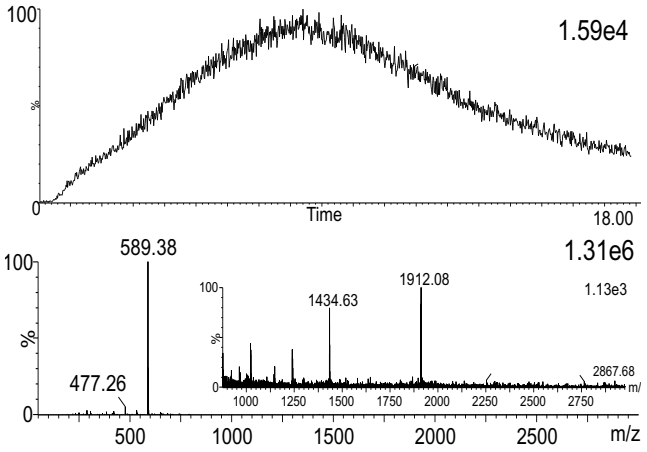


Figure 3: TIC and mass spectrum of bovine insulin using 3-nitroaniline as the vMAI matrix. The sample was introduced to the vacuum of a Waters SYNAPT G2S mass spectrometer using probe introduction. The maximum ion abundance of insulin is about 400X less than with coumarin, however an intense signal is observed for *m*/*z* 589. This signal may represents a compound which ionizes well with the 3-nitroaniline matrix.

expanded inset. However, an ion at *m*/*z* 589 dominates the mass spectrum of 3-nitroaniline with an abundance of *ca.* $1e^6$. At this time, this ion is an unknown, which is either in high concentration in the matrix, or it is efficiently ionized by this matrix. Either way, it demonstrates that even though the analyte insulin is ionized in poor abundance, ions are produced in high abundance with this matrix.

Testing matrices against a wider array of compound classes may result in discovering additional useful vMAI matrices. Even with vMAI matrices that were shown to be useful for a mixture containing a peptide and small protein, differences in ionization is still noted. As an example, we acquired data from a >10 year old sample labeled 'Waters BSA tryptic digest, 1 pmol μL^{-1}' using two vMAI matrices, methyl 2-methyl-3-nitrobenzoate and 3-NBN (**Fig. 4**). The mass spectra were acquired with the MS^{TM} prototype high throughput vMAI source on the Q-Exactive Focus. As expected, the 3-NBN matrix provided higher ion abundances, although the most abundant ion at *m*/*z* 663 in the spectrum using 3-NBN is commonly observed with this matrix. While many of the ions observed in the two mass spectra had the same mass (shown in purple), their relative abundances were different with the two matrices indicating different ionization efficiencies. Most of the multiply charged ions represent peptides that are found in both mass spectra in some charge state indicating that both matrices work well with peptides, although this is not universally true as seen by the triply charged ion at *m*/*z* 1259 (MW

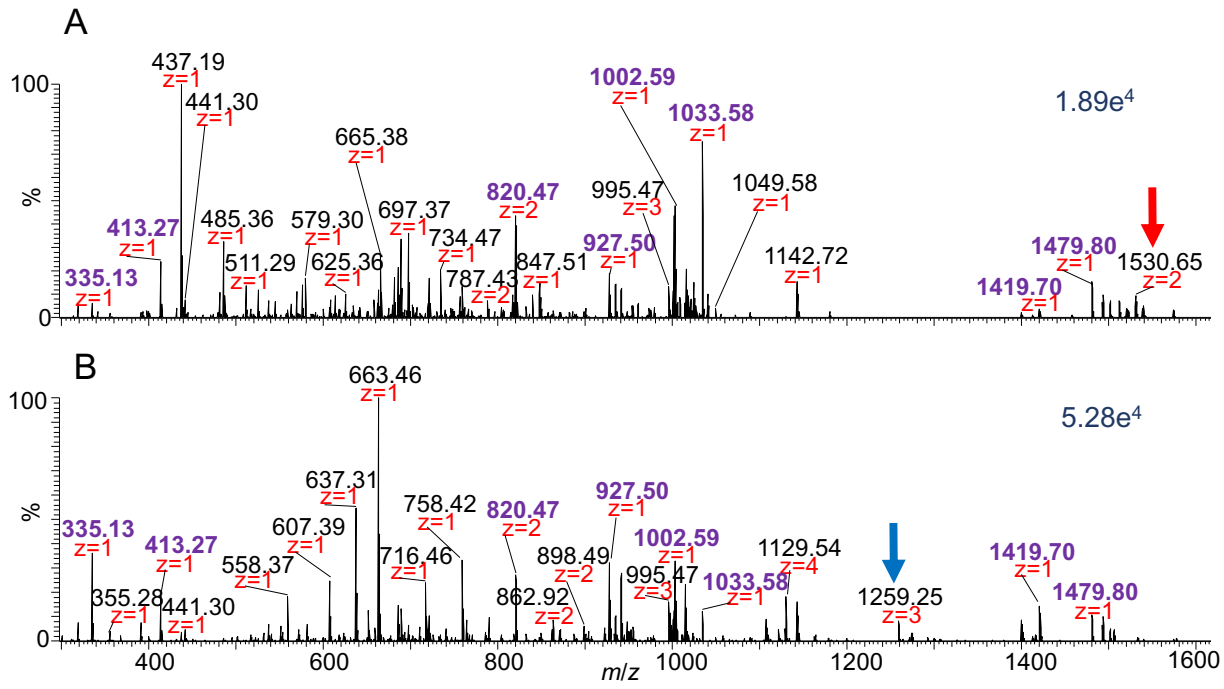


Figure 4: vMAI mass spectra of a >10-year-old sample labeled "Waters BSA Tryptic Digest, 1 pmol μL^{-1}" using A) methyl 2-methyl-3-nitrobenzoate and B) 3-NBN as matrices. The spectra show identical ion labeled in purple, as well as, differences labeled in black. Multiply charged ions which are different in the two mass spectra are labeled by arrows. The mass spectra were acquired on a Thermo Q-Exactive Focus using the MS™ prototype high throughput vMAI source.

3774) with 3-NBN and the doubly charged ion at *m/z* 1530 (MW 3058) with the benzoate matrix. We find no other charge state ions in the opposing mass spectrum representing these molecular weights. However, a number of the singly charged ions are observed in one, but not both mass spectra even after eliminating ions associated with the matrices.

Manipulating Ionization With Binary Matrices

In MALDI and ESI, the means of producing gas-phase charged particles/droplets seems obvious, but in vMAI neither sublimation nor solvent evaporation are associated with expulsion of particles into the gas phase. Chapman, in 1938, demonstrated that boiling water produces gas phase charged droplets,[47] and ionization during sublimation may involve a similar chaotic process whereby charged matrix particles are expelled into the gas phase. SAI, in which the matrix is a solvent, involves passing solution droplets through a capillary from atmospheric pressure into vacuum.[22] In such a process, evaporative cooling, depending on the solvent, can produce freezing, which also produces analyte ions, at least from water, methanol, or mixtures thereof.[38] However, if the droplets passing through the capillary remain liquid, possibly because the capillary is heated, they will reach a pressure in which superheating occurs with the potential for violent boiling and release of charged droplets.[22,48] With a subliming matrix, favorable conditions, such as placement

under certain vacuum conditions,[4,7] or even heating the matrix under atmospheric pressure conditions,[49] might also provide the chaotic environment needed to expel particles into the gas phase.

One means of providing a more chaotic environment is to add a more volatile component to the matrix. This was accomplished with SAI by carbonating the water used as the solvent.[50] Under the same conditions of analyte concentration and inlet temperature, the carbonated solution consistently gave higher ion abundance than still water under identical infusion rates. With vMAI, adding the 3-NBN matrix containing about 25% methyl-2-fluoro-5-nitrobenzoate or methyl-3-nitrobenzoate, compounds that sublime faster than 3-NBN alone, to a 500 fmol μL^{-1} water solution of gramicidin S produces maximum ion abundance faster than with 3-NBN. These matrix additives alone are ineffective at ionizing gramicidin S, but combined with 3-NBN they do not decrease the total ion abundance, and because the ion current is observed over a shorter duration, the maximum ion abundance relative to 3-NBN is increased. These binary matrix mixtures have two advantages; 1) the maximum ion abundance is reached faster which is helpful for fast high throughput analyses, and 2) because the matrix mixture sublimes faster than 3-NBN, the time ionization is observed, and thus carryover, is shortened. Using 0.2 µL the 1:1 gramicidin S:3-NBN solution dried on a glass slide, and the MS™ prototype manual high throughput vMAI source, we observe the doubly charged ions of gramicidin S for *ca.* 14 sec with maximum ion abundance achieved after *ca.* 9 seconds (**Fig. S5**). Using the binary matrix containing 15% of methyl-3-nitrobenzoate, the total duration is 3.6 sec and maximum ion abundance from first exposure to vacuum is *ca.* 3 seconds (**Fig. S6**). Further, the maximum ion abundance is over 4X greater with the binary matrix. We hypothesize that sublimation of pockets of the rapidly subliming matrix compound increases the 3-NBN surface area which in turn increases the rate of sublimation and thus the rate analyte ions are formed. Matrices such as coumarin and methyl-2-methyl-3-nitrobenzoate sublime faster than 3-NBN and also have less delay time before peak ionization. The duration of ion abundance is likewise reduced with these more volatile matrices.

An interesting example of a binary matrix improving the ion abundance of lysozyme, but not ubiquitin, was achieved by the addition of α-cyano-4-hydroxycinnamic acid (CHCA) to 3-NBN (**Fig. 5**). CHCA is nonvolatile under the conditions of this experiment so that sublimation cannot be responsible for the result. One possibility is that CHCA interacts with lysozyme to reduce aggregation as the solvent dries to form the matrix crystals. We speculated such a role for CHCA may account for its use in MALDI for protein analysis. However, sinapinic acid, another matrix used in MALDI for protein analysis, when mixed with 3-NBN reduces the ion abundance observed for lysozyme. Another binary matrix example of

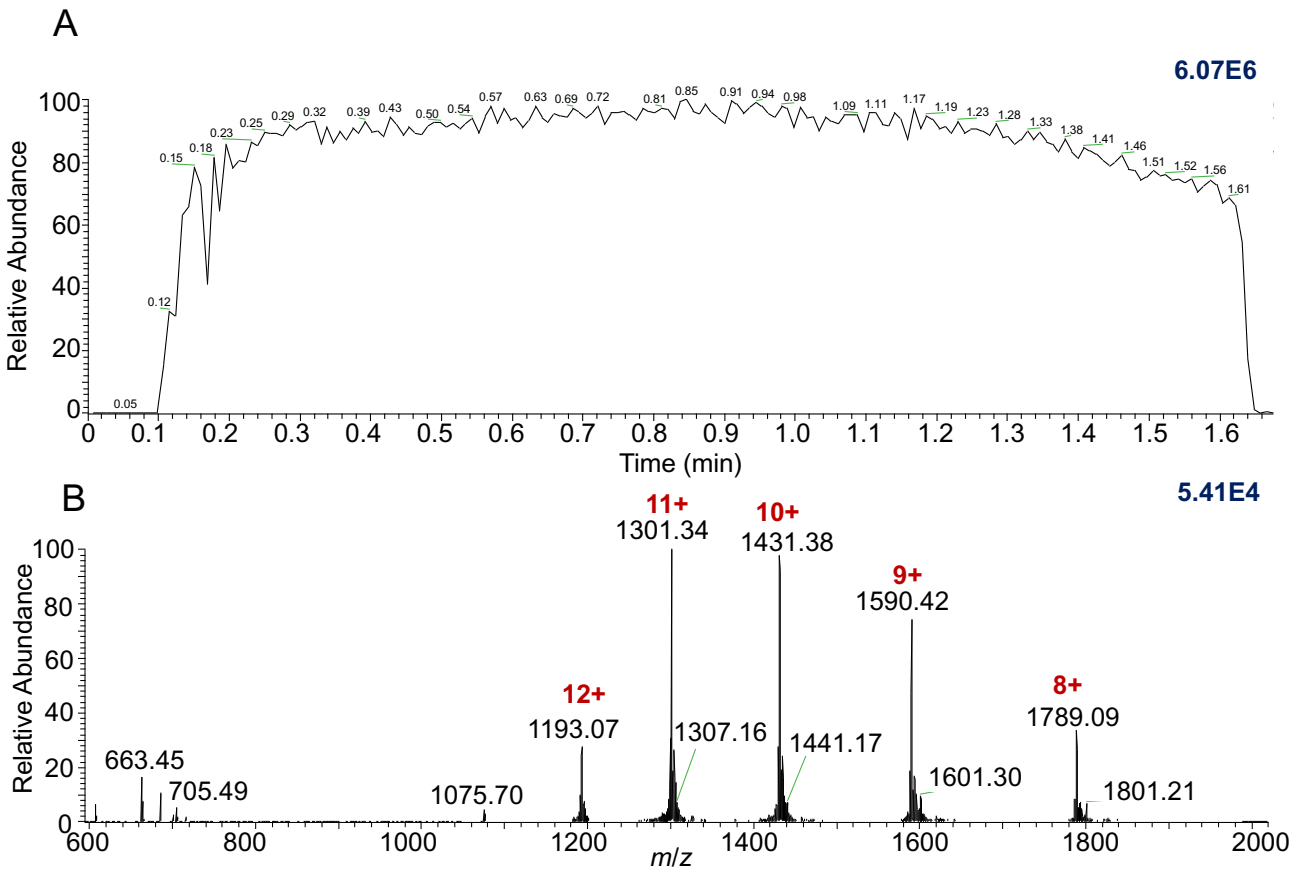


Figure 5: vMAI mass spectra of 1 pmol of lysozyme acquired on a Q-Exactive Focus using the MS^{TM} prototype high throughput vMAI source with the binary 3-NBN/CHCA matrix. The addition of CHCA to 3-NBN improves the mass spectra relative to 3-NBN alone as matrix.

improving ion abundance is mixing 1,3-dicyanobenzene (DCB) and 1,3-dinitrobenzene (DNB) to form a binary matrix mixture using vMAI on the commercial vacuum MALDI source without engaging the laser of the SYNAPT G2S.[31] Nearly an order of magnitude increase in ion abundance of analytes consisting of 3 pmol μL^{-1} each of fexofenadine (MW 501) and azithromycin (MW 748), and 5 pmol μL^{-1} each of angiotensin I (MW 1295) and insulin (MW 5733) were observed with the mixed matrix combination of 3 parts DCB to 2 parts DNB relative to using the 3-NBN matrix alone. Interestingly, DNB and DCB alone as matrices gave about 2 orders of magnitude less ion abundance than the mixture, and neither matrix produces gas phase ions from the insulin component. Because of the important implications of the results with the DCB/DNB binary matrix, the studies were repeated on the Q-Exactive Focus using probe introduction. Similar results were observed in that for DCB only a low abundance triply charged ion of insulin was observed and the base peak was the doubly charged ion of azithromycin with ion abundance of $6.7e^4$ (**Fig. S7**), and for DNB low abundance insulin ions were observe with the base peak being the singly charged ion of fexofenadine with ion abundance of $6.9e^3$ (**Fig. S8**). A 1:1 molar mixture of the matrices produced

abundant ions for all compounds in the mixture with base peak ion abundance of $4.2e^5$ with insulin showing charge states for +3 to +6 in high abundance (**Fig. S9**). The matrices 3-NBN, DCB, and DNB have in common that either nitro, cyano, or both groups are in the meta position of benzene, therefore, it would appear that structural motifs might be critical to the success of vMAI. However, a look at the structures of the matrices in **Scheme 1**, and results for *e.g.*, coumarin and phthalic anhydride in **Figure 1** do not seem to confirm this, similar to reports for MAI matrices.[5,6,8] Clearly, different matrix structures result in different analyte ionization efficiency. Additionally, matrix properties can be manipulated through the use of additives such as ammonium salts,[51] azo compounds,[30] and binary matrices reported here.

As noted above, in order for a charged particle to separate from the surface, it must overcome an induced electrostatic charge making the process more energy intensive than expulsion of neutral particles, thus raising the possibility that neutral particle expulsion is more general for subliming compounds than production of charged particles from which gas phase ions are produced. Minimizing such a process is important for sublimation purification. The expelled particles, although likely nanometer in size, are still more massive than the sublimed matrix molecules, and thus with a properly designed sublimation device may not be able to travel to the collection area. On the other hand, for MS, increasing the number of charged particles expelled from the surface is expected to result in improved sensitivity. Because this relatively new ionization process is already of comparable sensitivity to ESI or MALDI,[9,26,31,46] it represents an excellent opportunity to further decrease the limits of detection using MS.

Conclusion

Considering the MAI process occurring in vacuum (vMAI), energy considerations eliminate bare ions desorbing from a surface,[19] and there is no mechanism for gas phase ionization. Such desorption processes are even more unlikely considering no external source of energy is required for even proteins to be transported into the gas phase as multiply charged ions. The most plausible means for achieving gas-phase ions is through a charged matrix:analyte cluster/particle mechanism. Experiments point to sublimation as being required for observation of gas-phase ions, but sublimation, like evaporation, is a molecular process without an obvious means of expelling charged particles into the gas phase. However, because ionization under vacuum conditions is a continuous process, similar to ESI, which stops when sublimation ceases, there must be a link between particle emission and sublimation. An attractive possibility is a chaotic process akin to liquid boiling but occurring during sublimation just as evaporation occurs during boiling. Even so, experiments show that sublimation is the dominant process with vMAI matrix compounds when

exposed to vacuum conditions inside a mass spectrometer, and ion emission is a minor process[8] possibly explaining why the process has not previously been reported. Even though only a small fraction of the nonvolatile compound enters the gas phase as ions, when used as an ionization process for MS, the method has exceptionally high sensitivity.[3,5,52] These results imply that the sublimation process in some cases is more involved than just molecules leaving a surface, and that enhancing the alternative process(es) occurring during sublimation might positively impact the sensitivity achievable using MS and poorly effect sublimation purification processes

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

The Supplemental PDF file contains a description of ion yield calculations for MAI as well as mass spectra using various MAI matrices and binary matrices.

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Author Contributions

The manuscript was written through contributions of all authors. / All authors have given approval to the final version of the manuscript.

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