New Ionization Processes for Ionizing Nonvolatile Compounds in Mass Spectrometry: The Road of Discovery to Current State-of-the-Art

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ABSTRACT: This *Perspective* covers discovery and mechanistic aspects as well as initial applications of novel ionization processes for use in mass spectrometry that guided us in a series of subsequent discoveries, instrument developments, and commercialization. With all likelihood, *vacuum* matrix-assisted ionization on an intermediate pressure matrix-assisted laser desorption/ionization source *without*

the use of a laser, high voltages, or any other added energy was the defining turning point from which key developments grew that were at the time unimaginable, and continue to surprise us in its simplistic preeminence, and is therefore a special focus here. We, and others, have demonstrated exceptional analytical utility without a complete understanding of the underlying mechanism. Our current research is focused on how best to understand, improve, and use these novel ionization processes through dedicated platform and source developments which convert volatile and nonvolatile compounds from solid or liquid matrices into gas-phase ions for analysis by mass spectrometry using *e.g.*, mass-selected fragmentation and ion mobility spectrometry to provide reproducible, accurate, and sometimes improved mass and drift time resolution. The combination of research and discoveries demonstrated multiple advantages of the new ionization processes and established the basis of the successes that lead to the Biemann Medal and this *Perspective*. How the new ionization processes relate to traditional ionization is also presented, as well as how these technologies can be utilized in tandem through instrument modification and implementation to increase coverage of complex materials through complementary strengths.

Introduction

Our research broadly relates to developing technology for molecular characterization of surfaces to provide spatially resolved chemical information of biological and synthetic materials using mass spectrometry (**MS**). While surface chemistry is economically extremely important, molecular methods of surface characterization, including the molecular structures of organic and inorganic compounds in surface layers, healthy *versus* diseased tissue, *etc.* lag behind materials characterization in solution. Lack of methods with high mass and spatial resolution, as well as sensitivity capable of characterizing complex surfaces and surface reactions are limiting developments in surface characterization, clinical chemistry, and ultra-fast analyses.

The accumulation of discoveries, developments, and implementations of new ionization processes for use in biological MS was honored with the Biemann Medal at The American Society for Mass Spectrometry (**ASMS**) meeting in 2019.[1] Together with collaborators from academia, government, and industry, key limitations of materials characterization have been identified for which current analytical technology are insufficient. Material characterization is an essential component of materials developments, clinical precision, and safety, and, consequently, commercialization. The objective of our work is to improve the utility through simplicity and sensitivity of the new ionization processes, with some emphasis on *vacuum* matrix-assisted ionization (***v*MAI**),[2,3] in order to

broaden the scope of materials for which molecular surface and chemical defect analyses can be applied. Recent summaries include research on fundamentals and applications and, in some cases, are supported by movies showcasing the operation of different methods.[4–10] In this *Perspective* we present work delivered in the Biemann lecture in June 2019[11] and previous ASMS presentations from us and key collaborators to demonstrate scientific and broader societal impact. We first start with the end, the state-of-the-art technologies. Subsequently, we include a synopsis of discoveries going from the solvent-free and voltage-free experiments to the dedicated sources presenting *v*MAI as the newest, simplest, and most robust alternative within these new ionization processes, as well as the lessons learned toward taking informed steps through hypothesis-driven fundamental research questions guiding and enabling the discoveries to advance the new technologies and applications. To assist the reader, **Scheme 1** summarizes the path of the discoveries and inventions, how they are interconnected fundamentally and how they have been used by us and others, as well as the homebuilt modifications to commercially available manual and automated platforms (*inlet* ionization) and sources (*vacuum* ionization).

Experimental

Details are included in the SI of this contribution.

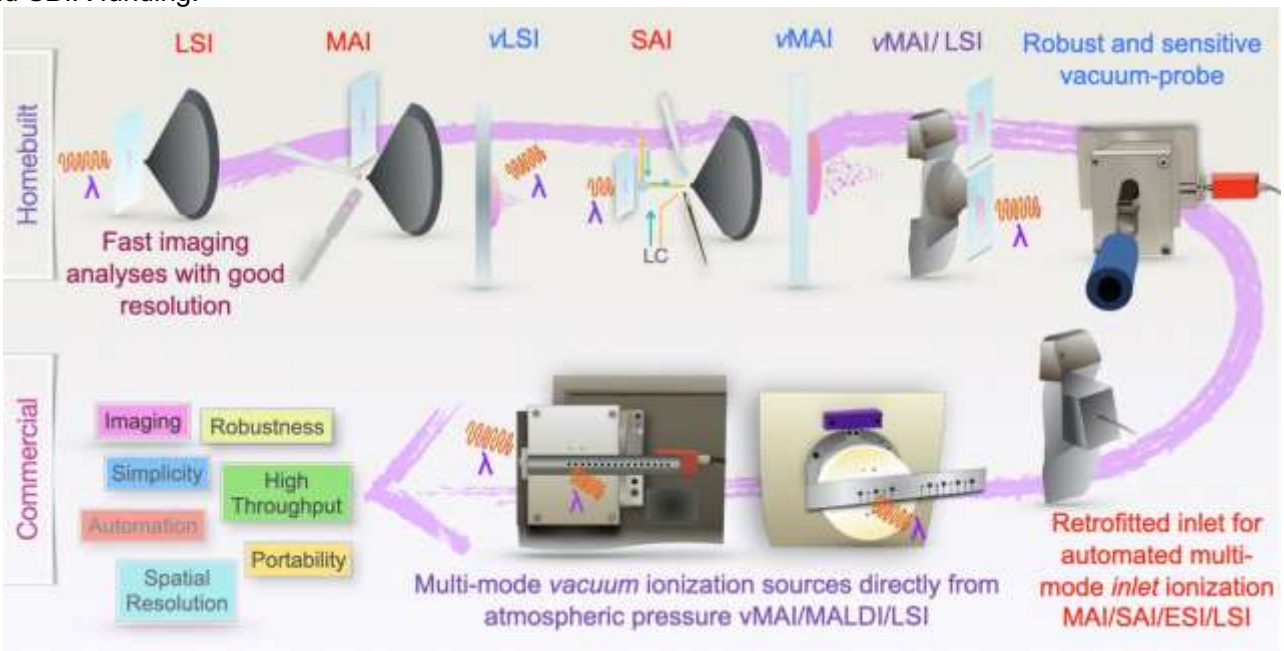
Results

1. State-of-the-Art Technologies for Fast, Sensitive, and Robust Analyses Without Mass Spectrometry Expertise

We start this *Perspective* at the current state of development and then describe the important progression from its inception, bringing us full circle from the first observation of vMAI spontaneously producing gas-phase ions simply by exposing the matrix:analyte sample to the vacuum of the mass spectrometer. Briefly, this was accomplished on Waters SYNAPT G2 and G2S intermediate pressure matrix-assisted laser desorption/ionization (**MALDI**) sources and required 2 minutes to insert the sample into the ion transfer region. Because vMAI requires matrices which sublime in vacuum, we needed a faster means of delivering the sample. This was accomplished in the Trimpin academic lab at Wayne State University (**WSU**) with a probe inserted through a ball valve providing *ca*. 10-second introduction. The results, as described below, were so enticing that MS™, a university spin-off company between Wayne State University (**WSU**) and the University of the Sciences, founded to commercialize the new ionization technologies, built a probe device to test on a Thermo Orbitrap mass spectrometer. The results, especially from the second-generation probe device were astonishing in sensitivity, robustness to carryover, and, theoretically at least, robust to instrument contamination as described in more detail later on. While the Trimpin group fo cused on fundamentals of the ionization process and discovery

of new matrices which had been too volatile to survive the time constraints of the vacuum MALDI source introduction (described later), MS™ focused on achieving automated high throughput, while maintaining all the advantages of a vacuum-probe.[12,13] In order to give the reader a feel for the analytical power of the new ionization technologies described herein, we begin with the developments relative to high throughput vMAI.

Scheme 1: The principal timeline of the path of discoveries and inventions of new ionization processes developed into new ionization methods with applicability ranging from imaging to liquid chromatography and spanning pressure regimes from above atmospheric to low pressure (high vacuum) with the preferable operation directly from atmospheric pressure (**AP**) into the sub-AP of the mass spectrometer. *Inlet* ionization (in red color) is defined when the ionization is assisted by an inlet tube, preferably heated, that connects a higher pressure with a lower pressure region, by default available with AP ionization mass spectrometers. Depending on the vendor, mass spectrometer specific ion source configurations can be used "as is" or with *inlet* modifications. In matrix-assisted ionization (**MAI**) a laser is not used to initiate ionization of the matrix:analyte sample introduction in the mass spectrometer inlet, and in solvent-assisted ionization (**SAI**) the matrix is a liquid, typically the solvent used to dissolve the analyte. *Vacuum* ionization (in blue) is defined when the matrix:analyte is introduced into the sub-AP of the mass spectrometer where ionization is spontaneously initiated. Vacuum LSI (*v*LSI) proved that a heated inlet tube was not necessary instead, multiply charged ions are obtained using the energy of a laser. Without the use of a laser but using the matrix 3-nitrobenzonitrile under vacuum conditions (*v*MAI) was discovered. Using vMAI, the changes in the instrument can be as minimal as with *inlet* ionization, or substantial by replacing the source and inlet to the mass spectrometer. The purple color combines substantially the aspects of inlet and vacuum ionization. MS™ products include manual and automated Ionique platforms and sources through the National Foundation Science (**NSF**) STTR and SBIR funding.



The performance of the manual vacuum-probe on the Q-Exactive Focus[13] convinced MS™ to apply for and receive an NSF SBIR Phase I award to develop a high throughput vMAI source using a novel sample introduction approach. The success of the probe sources, even though limited

to single sample insertion, eventually inspired a new concept for sample introduction to vacuum

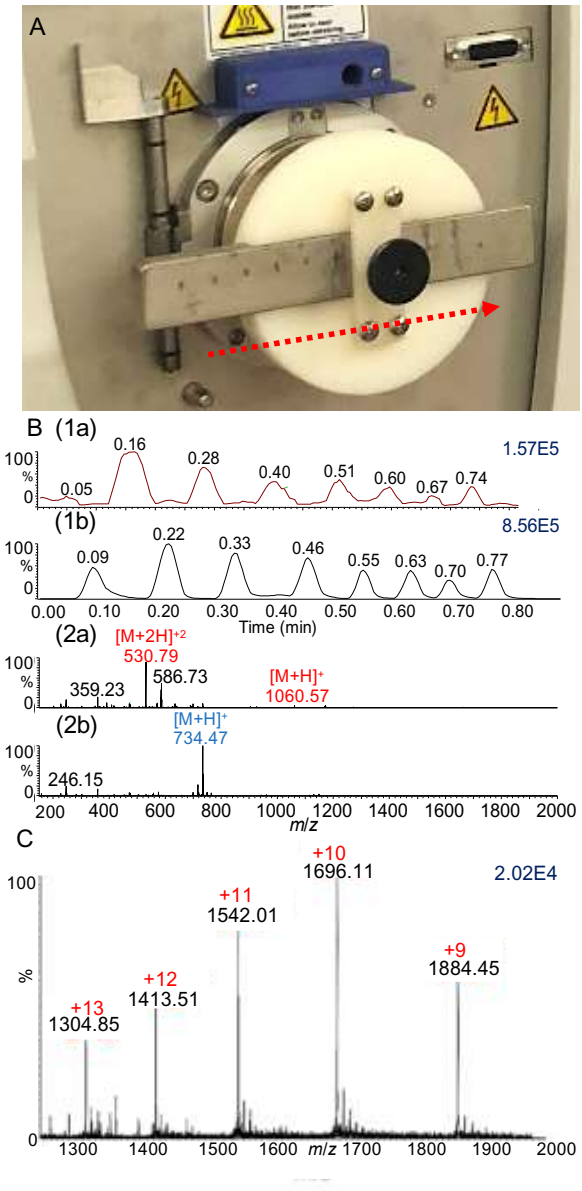


***Fig. 1**: Sequential sampling by vMAI-MS directly from AP into sub-AP of the mass spectrometer, (**A**) photograph of the source set-up using a plate introduction in which each matrix:analyte sample is sequentially exposed to the sub-AP in short successions; red arrow shows the direction of the sample analysis; (**B1**) chronograms displaying 8 samples run sequentially in less than 4 seconds per sample and (**B2**) mass spectra of the two compounds sequentially exposed to the sub-AP of the mass spectrometer: (**a**) bradykinin and (**b**) azithromycin, (**C**) mass spectrum of the multiply charged ions of myoglobin MW = 16.7 kDa. First results were presented in the Biemann Medal lecture at the ASMS conference in Atlanta, Georgia.*

and has the potential for sensitive, robust, and high throughput analysis using vMAI (**Fig. 1**). Indeed, the first example of using this new ion source concept for rapid mass measurements was presented in the Biemann Medal lecture by Trimpin.[11] MS™ has demonstrated vMAI analysis speeds of 4 sec/sample using 8-well sample plates including the time required to load samples.[14] Because this vMAI concept, in theory, can be applied to any atmospheric pressure ionization (**API**) mass spectrometer, capabilities of high mass resolution, accurate mass measurement, MS/MS, and ion mobility spectrometry (**IMS**)-MS technology will be available for structural characterization studies.

Although a laser adds complexity and costs, MS™ also expanded the *vacuum* ionization source to include vacuum (v)MALDI (**Fig. 2A**). As it was shown in Dr. Sarah Trimpin's ASMS 2020 online presentation and the subsequent papers delivered in this Special Issue,[14,15] the new platform combines both technologies, vMAI and vMALDI, giving either singly (vMALDI) or multiply charged (vMAI) ions with the same mass spectrometer and no need of waiting time for the sample to be introduced to vacuum through the vacuum interlocks. Up to 8 samples can be loaded on a glass slide with sequential sample analysis simply by sliding the glass slide affixed to a spacer plate with 8 channels over a hole in a flange to the vacuum of the mass spectrometer. The sample and spacer plates remain at atmospheric pressure (**AP**) while one sample at a time is exposed to vacuum.

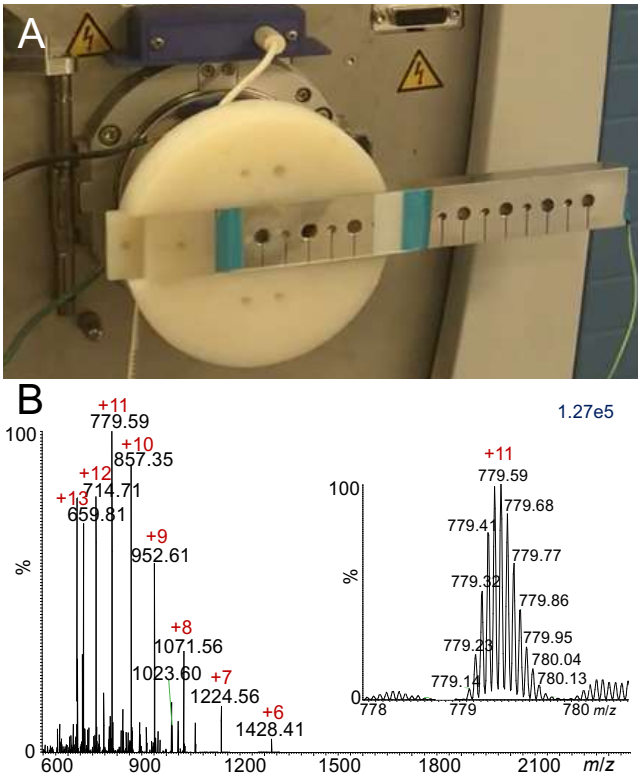


***Fig. 2**: Multi-mode vacuum ionization source: (**A**) photograph of vMAI/MALDI; (**B**) vMAI analysis of a mixture of the protein ubiquitin (5 pmol μL^{-1}) in the presence of a commercial BASF detergent and directly from a surface: mass spectrum with Inset: isotopic distribution of the charge state +11 ion at m/z 779.59. The detergent was used without dilution to which ubiquitin was added to obtain a final concentration of 10 pmol μL^{-1} of the protein. 0.5 μL of the mixture solution was spotted on a glass plate and dried for ca. 1 h to ensure complete dryness after which 0.5 μL of binary matrix (3-NBN:CHCA in a 2:1 v:v ratio) was added. The plate was directly placed from AP to sub-AP on the multi-mode ionization source operated in the vMAI mode. The details of the multi-mode source of vMAI/vMALDI are provided in ref. 11. The acquisition was in a time frame of a few seconds. Movie **S-01** is included to demonstrate the ease and effectiveness of this prototype source on the Orbitrap Q-Exactive Focus at MS™. Ref. 11 describes in detail the use of the multi-ionization source; in case of vMALDI the laser is aligned in transmission geometry (TG using an aged, inexpensive N_2 laser at 337 nm laser wavelength). The implementation of this source concept on a Waters SYNAPT G2S is provided in **Fig. S1**.*

Vacuum is maintained by having flat plates slide over the flat flange surface. Ionization commences with vMAI when a sample experiences vacuum and with vMALDI when the matrix:analyte sample is ablated by the laser beam.

With vMALDI, analysis speeds of one sample per second with full mass range acquisitions have been achieved.[14,16] With vMAI speeds are currently at the order of 3 seconds per sample. In an interesting example of the complementary nature of the two ionization methods, polyethylene glycol (**PEG**)-2000 and ubiquitin were mixed and applied on adjacent positions on a glass slide, one sample using 2,5-dihydroxyacetophenone (**2,5-DHAP**) as matrix, and the other 3-nitrobenzonitrile (**3-NBN**). A nitrogen laser was used to ablate the 2,5-DHAP prepared sample (vMALDI), and the 3-NBN sample *spontaneously* produced gas-phase ions (vMAI). With 3-NBN multiply charged ions of ubiquitin were observed, but no PEG ions, and with 2,5-DHAP, singly charged ions of the PEG sample were observed but no ions from ubiquitin.

The multi-mode *vacuum* ionization source of vMAI and vMALDI uses a flange component that is rather simple in concept as it consists of the flange having a flat surface. Details are included in ref. 14. This new vMAI source was also used to investigate the applicability of analyzing *e.g.,* proteins in the presence of detergents in collaboration with Dr. Vladislav Lobodin (BASF Corporation). Highly charged ubiquitin ions out of a variety of different detergents, without dilution, have been analyzed in a straightforward manner (**Fig. 2**). **Movie S-01** is included to visualize this point,

as it is nothing short of astonishing!

MS^{TM}'s current aim is to build an ultimately simple, highly robust, high throughput, automated source based on vMAI and vMALDI. Meanwhile, the Trimpin group has implemented the vacuum source to optionally include vMAI, vMALDI, and vLSI to replace the Waters source (**Fig. S1**). This next generation multi-mode ionization source, ideally available on all vendor mass spectrometers, has the potential to be transformative in how direct ionization is achieved with high speed, sensitivity, and robustness with the full scope of capabilities of state-of-the-art mass analyzers, including tandem mass analyzers, with collisional induced dissociation (**CID**), electron transfer dissociation (**ETD**) and other fragmentation technologies. Such developments could not have been imagined just a few years ago. In the next sections are described how we systematically developed the new technologies based on new ionization processes.

Lesson 1:

- A novel ion source makes *vacuum* ionization as simple to use as ambient ionization, but with unprecedented speed, and robustness
- Multi-mode ionization sources are cost-effective and analytically exceptional in information coverage, as shown in more detail in ref. [14]
- Exciting new fundamental research can now be performed to decipher the basics associated with the new ionization processes because the same mass spectrometers can be used under well-functioning conditions for the specific method of interest. Initial progress is detailed in ref. [15]
- Direct chemical, clinical and biological applications, for rapid and sensitive analyses

2. Straightforward Implementation of Proposed Imaging MS using Transmission Geometry Laser Ablation and Unexpected Observation of Multiply Charged Ions

Looking back, the start of all these ionization inventions was a relatively trivial idea to improve tissue imaging. The hypothesis was that high resolution and fast imaging MS directly from AP could be improved using transmission geometry (**TG**)-MALDI. Solvent-free sample preparation was used to minimize alteration to the tissue and maximize the chemical information as well as minimize the time of analysis to better study and understand complex organs such as brain function.[17] TG-MALDI had been performed previously at vacuum and AP.[18,19] A "field-free TG-AP-MALDI" proposal by Trimpin eliminated the voltages applied to extract the ions to overcome so-called "rim losses"[20,21] as well as offering simplicity to the set-up and experimental execution (**Fig. S2**).[17] Unlike previous TG-MALDI results, initial voltage-free experiments produced reasonably abundant analyte ions almost immediately from small molecules such as drugs and lipids using the MALDI matrix 2,5-dihydroxybenzoic acid (**2,5-DHB**).[17] Elimination of voltage was achieved by placing the point of ion production close enough to the inlet of the mass spectrometer in which the ablated material is flow entrapped into the sub-atmospheric pressure (**sub-AP**) of the mass spectrometer vacuum. Using the laser in TG, the physical closeness is readily achieved between

the point of ablation and the mass spectrometer inlet. Unexpectedly for a MALDI-like setup, multiply charged ions of peptides and even proteins were obtained (**Fig. S3**).[17] Only in the presence of high voltages were singly charged ions observed, similar to reports from Galicia and collaborators involving the use of voltage,[18] additionally to the multiply charged peptide ions.[17,22–25] Undesired metal cation and matrix attachments to the singly charged ions, and an increase in chemical background, were observed using higher voltages. The use of solvent-free matrix preparation led to ease in sample preparation and potential improvements in imaging experiments[17,26] but also the observation of only singly charged ions.[22] This was also the first indication of the importance of the solvent, especially the aqueous solution component, in these and likely many other ionization processes relative to the formation of multiply charged ions.

The implementation of MS imaging was readily a success[27] for singly and even for the surprising case of multiply charged ions using Thermo mass spectrometers.[28,29] Fast single-shot-one-mass spectrum output[17,26] along with multiply charged ions nearly identical to electrospray ionization (**ESI**) from a MALDI-like experiment were observed initially using the MALDI matrix 2,5-DHB. Spatial resolution of *ca.* 20 μm was achieved.[4,26,27,30] In brief, an automated X,Y,Z-stage was used in imaging tissue sections[4,28,29] to move the sample plate relative to the inlet aperture of the mass spectrometer and laser beam to obtain a series of mass spectra from which an image can be constructed for any mass-to-charge (m/z) value. Although the distance to the inlet can be relatively short (~1 mm) using the TG laser alignment, one might expect loss of ablated material other than to the mass spectrometer inlet. Different size inlet apertures were tested relative to ion abundance in collaboration with Dr. Alexander Makarov (Thermo) and Dr. Mike Morris (Waters). Even a 1-m long extended stainless steel inlet tube provided ions of the analyte.[5] There are numerous applications for molecular imaging, the quality of the results and the spatial resolution are typically limited by sensitivity. An increase in sensitivity is known to be associated with vacuum MALDI,[31,32] although the ionization efficiency is substantially lower than with ESI.[33,34]

Initial fundamental research with a laser on an API mass spectrometer demonstrated the importance of inlet temperature,[23,35] as well as collisions with surfaces such as gases and obstructions in the production of ions, and subsequently to the term "laser (as in MALDI) spray (as in ESI) ionization" or LSI.[23,30] Because of the production of multiply charged ions originating from a solid surface, both CID and ETD from analyte ions could be demonstrated.[23,25,30,36,37] As one example, multiply charged myelin basic protein (**MBP**) peptide ions from mouse brain tissue were gas-phase sequenced for the first time by a LSI-ETD approach.[30]

Lesson 2:

- Fast imaging from AP with good spatial resolution using MS was achieved, but this outcome while useful is not transformative.
- The breakthrough was that ESI-like ions are observed from MALDI-like experiments.
- The mechanism is an important unknown.
- "If at first the idea is not absurd, then there is no hope for it." *Albert Einstein*[38]

3. Fundamentally Logical Arguments Leading to Discovery of a New 'Ion Source': The Heated Inlet Tube

Mass spectrometers and IMS-MS instruments that operate without a heated inlet tube were initially difficult to use with the new ionization processes providing only poor analyte ion abundance, even with retrofitted homebuilt inlet tubes.[24,27,37,39–43] An improvement in the ion abundance was accomplished using a 90° bent inlet tube, possibly forcing the material to collide with the surface,[39] however, the restricted airflow prohibited long term use for *e.g.*, imaging applications (**Fig. S4**). Key factors in obtaining analytically useful results early on were a heated inlet tube, collisions (**Fig. S5**), the type of matrix used, and the use of the laser.[,23,37,39,41,44,45] For example, using the MALDI matrix 2,5-DHB, and especially with the less volatile α-cyano-4-hydroxycinnamic acid (**CHCA**) and sinapinic acid (**SA**) matrices, a higher inlet tube temperature (>250 °C) was necessary to initiate ionization.[37,40] More volatile matrices showed more promising improvements. Switching from 2,5-DHB[46] matrix to 2,5-DHAP,[47] also a MALDI matrix (**Scheme S1**),[48] allowed ionization with lower inlet tube temperature.[42] Nevertheless, reliable ion formation, especially for imaging applications, remained a difficult task on the Waters instruments, commercially equipped with Z-Spray skimmers cone sources, even with 2,5-DHAP matrix.

At the time, we assumed the laser was responsible for the ionization, and thus a number of experiments did not make sense. The undergraduate student, Christopher Lietz, for example, reported that when he accidentally touched the inlet tube with the matrix:analyte on the glass plate, much better analyte ion abundance was obtained as compared to using a laser. The graduate student, Beixi Wang, accumulated results with a neodymium-doped yttrium aluminium garnet (**Nd:YAG**) laser provided by Dr. Arthur Suits (WSU). The laser used at 1060 nm, did not work well with the known MALDI conditions *e.g.*, glycerol, but when she used 2,5-DHAP (**Fig. S6**), which absorbs in the ultraviolet (**UV**), significantly better results were obtained. Essentially the same ion abundance, charge states, and drift times were observed at 1060, 532, and 355 nm with standards and mouse brain tissues provided by Dr. Ken Mackie's lab (Indiana University).[37,45]

Directly after a conversation between Trimpin and Dr. Charles N. McEwen (University of the Sciences) discussing these and other seemingly "odd" results, such as the ionization continuing after the laser was off, it appeared that the laser was

not responsible, nor necessary for ionization to occur. McEwen tapped a glass plate, containing the matrix 2,5-DHB and peptide analyte sample, against the heated inlet tube of a Thermo Exactive mass spectrometer without the use of a laser. He immediately called back with the news that the laser was, in fact, not necessary.[47] A flurry of experiments were initiated by both labs to introduce the analyte in a variety of new ways to the inlet of the mass spectrometer (vibrations, acoustic, punch, tapping, *etc.*)[5,11,22,49] and to better define mechanistic aspects, some of which were covered in previous papers.[5,7–10,37,50–52] In one example, the graduate student, Vincent Pagnotti, substituted the laser and glass microscope slide with a 'BB gun' and metal sample plate as a visual demonstration that almost identical mass spectra were obtained by both approaches.[49,53] The successful outcome of forming highly abundant multiply charged analyte ions, unequivocally demonstrated that the laser wavelength[35,53] has no influence on the experimental results other than delivering the matrix:analyte sample into the inlet tube of the mass spectrometer, and thus, explaining the previously "odd" results. Other examples of experiments to better define this new ionization method include various length and diameter inlet tubes, gas pressure and gas type (**Fig. S7**) with and without the laser, in the positive and negative modes as a means to improve the experimental outcome. These experiments in sum proved successful in increasing the analyte ion abundance, but none provided the desired breakthrough for analytical utility with mass spectrometers not already equipped with a heated inlet tube 'source'.[37,39,40] Consequently, a heated inlet tube used with some API mass spectrometers was an effective ion source, but the path forward,[51,54–56] has not always been straightforward.

The evolvement of the TG alignment for being possibly associated or responsible for the unusual observations of multiply charged ions was eliminated by using a MassTech AP-MALDI source, which uses the laser alignment in reflection geometry (**RG**), in collaboration with Dr. Barbara Larsen (DuPont).[47] Other experimental designs were tested to improve our understanding and the utility of the formation of multiply charged ions directly from surfaces. In 2013 McEwen's group demonstrated the use of LSI to simultaneously image lipids, peptides, small proteins and drugs.[57] The same year Dr. Rainer Cramer (University of Reading) and Dr. Klaus Dreisewerd (University of Munich) reported the use of liquid UV matrices for AP-MALDI, using a heated ion transfer tube on a Q-Star Pulsar AB SCIEX instrument, obtaining multiply charged ions of peptides and proteins.[58] In 2018, a miniature ion trap mass spectrometer was coupled with LSI by the Xu group (Beijing Institute of Technology) for the direct detection of peptides, proteins, and drugs in complex samples such as blood and urine, and lipids from tissues.[59] The same group, then adapted this setup to a 2-dimensional (**2D**) moving plat-

form for high throughput analysis (10 seconds per sample). Drugs, vitamin B, and peptides were analyzed in diluted blood, demonstrating the speed of 10 seconds per sample.[60] Direct bacterial analysis was demonstrated with this system; 21 bacteria were differentiated in both genus and species utilizing orthogonal partial least squares (**OPLS**).[61]

Lesson 3:

- Confirmation that the laser is not responsible for analyte ionization (therefore not "MALDI" but **MAI**)
- LSI is MAI with use of a laser to transfer matrix:analyte into the ionization region with high spatial resolution
- Confirmation that gas flow, collisions, and heat matter
- The research goal is to make LSI and MAI analytically useful

4. Expanding our Fundamental Knowledge of the New Ionization Processes Leading to Unique New Ionization Methods with and Without a Laser from Solids and Liquids

Studies that form key pillars in the path of our discoveries and innovative technology developments were made essentially at the same time. Both developments grew, at least in part, out of the success of the more volatile matrix 2,5-DHAP, relative to 2,5-DHB (for more detailed information about matrices see **Scheme S1**), that gave good results on the AP-MALDI source.[47] First, we describe developments of vacuum LSI (**vLSI**) in Trimpin's lab,[62] and second, solvent-assisted ionization (**SAI**) in McEwen's lab,[56] and from each, we briefly expand on the current uses and further developments by the community.

Using the commercial intermediate pressure MALDI source of a Waters SYNAPT G2 mass spectrometer and 2,5-DHAP as matrix gave exceptionally clean and highly abundant multiply charged ions upon laser ablation.[37,44,62,63] Hence, no inlet tube is necessary when using the 'right' matrix in what again seems like a MALDI experiment. Because of the formation of multiply charged ions, the mass range is extended and because of the fundamentals that were already established from AP, we used the term vLSI.[62] Our hypothesis that an important difference between LSI and MALDI in forming ESI-like multiply charged ions is the volatility of the matrix has been shown by us, and others,[37,52,64] and is best seen with the invention of vMAI (described below). An even better performing matrix, 2-nitrophloroglucinol (**2-NPG**) was subsequently discovered[44] and successfully used for the first imaging applications of several multiply charged MBP peptide ions from a mouse brain tissue using an intermediate pressure MALDI source and the laser in RG alignment.[65] The applied laser fluences and voltages were kept low to increase the ion abundance of the multiply charged ions.[37,47,65] Because of the relatively high volatility of 2-NPG, but the good ion abundance of multiply charged ions, we decided to have a mixture of matrices, adding 90% of 2,5-DHAP and 10% of 2-NPG, thereby reducing the volatility of the matrix composition and allowing

molecular imaging of tissue samples that intrinsically require longer acquisition times. IMS-MS was fundamental to enhance the separation of lipids, peptides, and proteins directly from the tissue.[62,65] These results were encouraging but still with the limitation of the unintentional matrix sublimation of 2-NPG.

We, and others, have shown the utility of the new ionization processes for surface analyses and imaging in the positive and negative mode, in RG and TG modes, and from AP and intermediate pressure for lipids, polymers, peptides, and small proteins. As an example, using vLSI, we developed methods to detect and image gadolinium-containing complexes synthesized in the lab of our collaborator, Dr. Matthew J. Allen (WSU). These complexes are developed for potential use as contrast agents for magnetic resonance imaging (**MRI**).[66,67] Traditional MALDI-TOF mass spectrometers were limited in analyzing these compounds. Using the commercial intermediate pressure MALDI-SYNAPT G2 with MALDI matrices either failed, or produced significant background, fragment ions, and less resolved ions as compared to the 2-NPG matrix, in terms of mass resolution (*e.g.*, MALDI 25,312 and LSI 35,711, **Fig. S8**), stressing the importance and broader applicability of this new matrix. Interestingly, a variety of complexes with different side chain functionalities were analyzed, and in some cases, gadolinium complexes associated with matrix ions. The interpretation of the formation of an ion consisting of matrix adduction to analyte was verified by MS/MS (**Fig. S9**). It is hypothesized that the nitro group of the matrix interacts as an additional ligand to the metal center.[68] Further studies will be required to understand the fundamentals as it may reveal important mechanistic information of the synthesized structures as well as the ionization mechanism using this and other matrices in MS.

Using an imaging approach, we analyzed mouse brain tissue sections in which the synthesized gadolinium(II) texaphyrin was administered prior to sacrificing the animal similar to other imaging work.[69] The 2-NPG matrix [below is says DHAP/NPG binary matrix] was spray-coated[65] onto the tissue section and the laser attenuated to sufficient analyte ion abundance, as is the case with any MS imaging experiment. The obtained MS images reflect the isotopic distribution of gadolinium with *m*/*z's* ranging from 917 to 925 (**Fig. S10**) by both the relative ion abundances of the isotopic distributions and the ion intensities of the images. This is contrary to *e.g.*, the typical lipids composition, in a similar *m*/*z* range. This relationship between isotopic *versus* image intensities serve as additional verification that this is indeed the synthesized gadolinium-containing product signal without the necessity of performing MS/MS for confirmation. No other gadolinium-containing isotopic distributions were obvious suggesting biological stability of the potential MRI contrast agent in biological applications. The location of this chemical within the mouse brain tissue section

matched well with that of the myelin areas of the brain.[69]

The matrix 2-NPG is to date one of the most valuable *vacuum* ionization matrices in conjunction with the use of a laser. High vacuum MALDI-TOF mass spectrometers were used to ionize and detect the highest charge states so far reported with a high vacuum mass spectrometer, and even in the reflectron detection mode.[52,64] Such results were highly sought after since the 1990s.[70–72] In 2014, Li's group (University of Wisconsin-Madison) reported on the use of LSI on an LTQ-Orbitrap XL mass spectrometer with an intermediate pressure MALDI source and CID as well as high-energy collision dissociation (**HCD**) for *in-situ* protein identification, visualization and characterization.[73] The 2-NPG matrix further enforced our hypothesis that multiply charged ions result from more volatile matrices being capable of rapidly subliming or evaporating from multiply charged gas-phase matrix particles containing analyte as discussed in a 2012 mechanism paper.[37]

The need for volatility to obtain multiply charged ions and the need for stability for long imaging experiments are counter to each other. Binary matrix combinations have somewhat been able to improve the stability without interfering too much with abundances of multiply charged ions. However, the key to successful imaging with more volatile matrices is to align the laser in TG for shortening the overall acquisition time (single-shot-one-mass spectrum to give a pixel). Initial successes have been reported by several groups using 2,5-DHAP and 2-NPG, respectively with vacuum MALDI instruments.[73–75] As a few examples, Caprioli's group (Vanderbilt University) demonstrated the utility of the use of TG-MALDI and 2,5-DHAP achieving protein images with 1 μm resolution.[76] Murray's group (Louisiana State University) included the use of two component matrix using 2-NPG and silica nanoparticles, producing high charged ions from tissues on a high vacuum MALDI-TOF.[77] In a recent study by Dr. Jeongkwon Kim's group (Chungnam National University),[78] 2-NPG compared with other MALDI matrices, demonstrated the ability to generate multiply charged ions of proteins such as bovine serum albumin (**BSA** +4 charge states) and immunoglobulin G (**IgG** +6 charge states). The addition of hydrochloric acid (**HCl**) enhanced the absolute protein peak intensities and the charge states become also more abundant,[78] as previously reported for new matrices in MS.[37,40,79] In the IgG study using linear detection mode, the typical wide unresolved peaks (*ca.* 10 kDa) associated with metastable ions, matrix and metal cation adductions, typical for a MALDI process, were observed.[80–83] In contrast, *intact* protein ions, *e.g.* BSA, were also detected highly charged (+14) in the reflectron mode, and the peak width (+9 charge state) had a more analytically useful signal width of *ca.* 600 Da.[64] The success is attributed to the softness associated with these LSI matrices as well as their general trend of not attaching matrix and

metal cations when forming the molecular ions, as evidenced with small molecules to smaller proteins.[37,40,44,64,65,84]

Another key pillar was the discovery of solvent-assisted ionization (**SAI**) in McEwen's lab[56] Vincent Pagnotti who succeeded in employing the solvent used to dissolve the analyte(s) as the matrix. Considering how often ESI is used in conjunction with a heated inlet tube, it is amazing this was the first reported example in which a solvent was introduced into an inlet tube to a mass spectrometer, preferably heated, to cause ionization of the analyte contained in the liquid.[49,56] In retrospect, this is a variation of MAI, in that a matrix is used but is in the form of a solvent instead of a solid phase compound. In an early example, the solvent with the sample is introduced using a silica capillary into the inlet tube showing multiply charged ions like ESI.[56,85] A variety of modes to introduce the samples followed and a few are summarized with photos (**Fig. S11**). Coupling SAI to liquid chromatography (**LC**)/MS was demonstrated, separating and ionizing components from tryptic digestion of BSA, as well as analysis of steroids with improved sensitivity relative to ESI.[86,87] High (*ca.* 100 μL) to low (*ca.* 0.2 μL) flow rates were applicable providing a stable spray.[56,86,88]

An automated X,Y,Z-stage was used to not only image tissue sections,[4,28,29] but also *e.g.*, in the automated analysis of well plates using the SAI approach. A motion-controlled arm was used to sequentially move an array of pipette tips containing the respective samples which were obtained from a well plate just prior to MS analysis. This approach readily detected the different sample solutions, and their relative concentrations were displayed using an ion intensity map (image of an *m*/*z* value of interest). The gas flow and sub-AP draws the liquid from the pipette tips into the inlet through space. This mist typically leads to 'splashing' and therefore undesired deposition on the outside of the inlet leading to cross-contamination, unless the use of pipette tips containing wash solvent are applied between acquisitions.[55]

The use of solvents as matrices is the basis of ESI and SAI. Solvents expand the range of matrices available to *inlet* ionization. McEwen's lab early on demonstrated a variety of unique introduction methods *e.g.*, a needle to sample an analyte solution,[49] as is demonstrated using the liquid matrix glycerol on an LTQ Velos with an inlet tube at high temperature (**Fig. S12A** and **Table S1**). Likely the easiest manner to ionize single samples has been the pipette tip, as is shown for the date rape drug gamma-hydroxybutyric acid (**GHB**) in the negative mode on an LTQ Velos at 50 °C (**Fig. S12B**). In most of the cases, though not surprising, the diverse variations of SAI have been accomplished using Thermo mass spectrometers that are already equipped with a heated inlet tube. During the ASMS Conference in 2011, McEwen's group introduced the method SAI-

surface sampling to analyze two and three-dimensional objects using a flow of solvent delivered to a sample under ambient conditions and then introducing the solvent/sample to the MS (**Fig. S13**).[49] The next year at ASMS Beixi Wang presented this system analyzing a drug-treated mouse brain tissue using continuous and discontinuous (discreet) flow.[88] An improved system using the manual Ionique platform, a syringe pump, and a gutted ballpoint pen was presented by our group in collaboration with MS^{TM} in Trimpin's ASMS talk from 2016 (**Fig. 3**). The pen held two fused silica capillaries together so that one delivered solvent and the other transferred the solvent to the inlet of the mass spectrometer where ionization occurs. Simply touching a surface of a sample (*e.g.*, tissue) and then collecting dissolved compounds using a liquid junction approach,[10] or by sequential transfer of defined extraction droplets to the inlet of the mass spectrometer allowed surface analysis of extracted compounds.[4,10,89] These approaches were easily performed on Thermo mass spectrometers with heated inlet tubes. For instruments without a heated inlet tube, it was initially most effective to construct an inlet tube to ionize nonvolatile compounds even from 99% aqueous solution.[43,49,90] Eventually we learned to analyze samples by SAI on Waters mass spectrometers, *e.g.*, a Waters SYNAPT G2 without a heated inlet tube[10,43,90,91] Inlet tubes varied in length from *ca.* 1 mm to 1 m with materials ranging from stainless steel, copper, and peak tubing.[4,5,10,36,39,41,87,89,92] In one example, a homebuilt heated inlet capillary (3" x

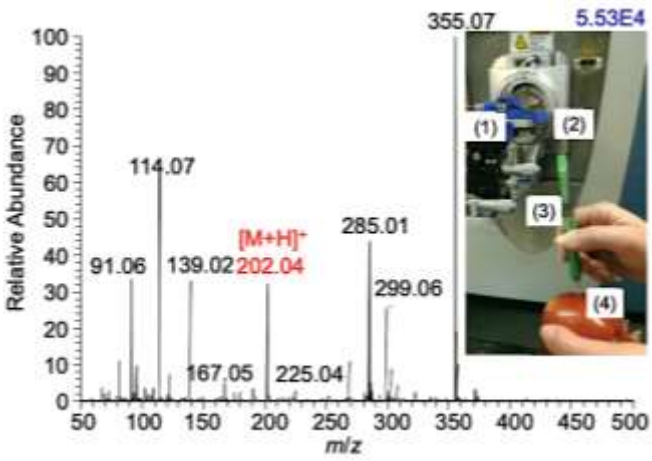


***Fig. 3**: Analysis of surfaces using MS Pen. The mass spectrum shows in red the pesticide thiabendazole m/z 202.04, the highest abundance of the pesticide was found in the stem area of the apple. Inset: photograph of the setup: (**1**) manual MS^{TM} platform with (**2**) fused silica capillary from the (**3**) MS Pen sampling of the (**4**) surface of an apple. Data acquired on Thermo Orbitrap Exactive mass spectrometer. See the complete description of the MS Pen in **Fig. S11C** and compare with **Fig. S13**.*

20 gauge) coupled to a Waters Xevo-TQ-S was used, and with this modification peptides and proteins were analyzed (**Fig. S14**).[90] Using more volatile solvents, and the addition of super-saturated gases into the solution,[93] the ion abundances increased on Waters instruments.

Different means to ionize and introduce liquid samples into the inlet of the mass spectrometer have been described by others. Johnston's group (University of Delaware) demonstrated the influence of an inlet tube heated to 950 °C by customizing the inlet cone of a SYNAPT G2S.[94] Their system uses an atomizer to generate droplets that later are introduced into the modified heated inlet

tube where the ionization process occurs; this method is called droplet-assisted ionization inlet (**DAII**).[94,95] Thermal bursting ionization is another method proposed to ionize molecules through a heating process. In this case, a liquid sample is added onto a heated metal probe where solvent is rapidly evaporated and the analytes are transferred from the liquid to the gas phase.[96] Another approach, a voltage-free ionization method presented by Drs. Stephen J. Valentine and Peng Li (West Virginia University), called "vibrating sharp-edge spray ionization" (**VSSI**), uses a piezoelectric transducer to generate an aerosol from the sample placed on a glass slide and placed close to the inlet tube of the mass spectrometer. The transducer is operated at low voltage input (12.7 to 30.6 V, peak to peak) and low power consumption (~150 mW)[97] compared with other ultrasonic[98] and surface nebulization methods.[99] In the VSSI approach, the high-frequency vibration at the sharp-edge is proposed to cause the detachment of small liquid droplets from the bulk fluid, resulting in a continuous spray.[97] More recently, the same group optimized the VSSI to be used as an interface to LC or other continuous flow-based applications by attaching a piece of fused silica capillary on the VSSI, called cVSSI,[100] resulting in the ionization of metabolites, peptides, and proteins comparable with LC-ESI-MS. This year cVSSI was improved to be a useful method for native MS in negative mode, delivering the liquid sample and providing a good signal with a minimal amount of sample at flow rates of 0.2 to 1 μL min^{-1}.[101] The newest study of this group is based on the understanding of the ionization mechanisms involved and the direct comparison with ESI using water and methanol as solvents.[102] The surface acoustic waves (**SAW**) processes have been used in the electronic industry[103] and more recently have been adopted in MS.[104–106]

Other groups are now working with *inlet* ionization methods for use as surface analyses tools, specifically with the help of a heated inlet tube. The advantage of this approach is the easy manipulation of samples (liquid or solid) outside the mass spectrometer. Surface analyses are increasing in popularity. One example with important clinical applications is the MasSpec Pen by Dr. Livia Eberlin's group (The University of Texas at Austin). A liquid junction is used in combination with SAI to detect cancerous tissue on a Q-Exactive Hybrid Quadrupole-Orbitrap mass spectrometer. Using a stop flow of water to sample the surface of the tissue, then taking advantage of the pressure differential, the sample/solvent is drawn into the instrument.[107] A modified MasSpec Pen is now integrated into the da Vinci surgical system[108] and was sold to Genio Technologies, Inc.[109] Following a similar path, the rapid evaporative ionization MS (**REIMS**) on a Thermo LTQ Orbitrap Discovery instrument and a Thermo LCQ Deca XP instrument was presented by Dr. Zoltan Takats' group (Imperial College London) in

2009.[110] In this paper, the authors detail that the electrosurgical ablation of the tissue is causing the ionization to occur before the transfer line so that the ions are transported towards the mass spectrometer, similar to desorption-ESI (**DESI**) remote sampling.[111] The ionization mechanism proposed is that ions may be formed by proton transfer reaction with the ionized water molecules, similar to AP chemical ionization (**APCI**). Alternatively, rapid thermal evaporation giving both the intact molecules and the fragments was proposed. In later work from this group, with Waters instruments, the inlet was modified to a heated capillary inlet in 2015 to improve sensitivity and robustness towards contamination.[112,113] More recently, they published matrix assisted (**MA**)-REIMS to improve the signal intensity in comparison with REIMS. In this case, the Waters instrument is configured with an inlet tube to the MS. Analyte in solvent droplets travel through the pressure differential in the inlet tube and exit near a collision surface that can be heated up to 900 °C.[114] Recently, the Takats' group presented another configuration coupling laser ablation to REIMS. This addition was applied to the metabolic fingerprinting of feces.[115] REIMS coupled with electrosurgery intelligent knife (iKNIFE),[116] was acquired by Waters Corporation[117] to develop applications in medical diagnosis. Kostiainen's group (University of Helsinki) presented the ambient method solvent jet desorption capillary photoionization (**DCPI**) which combines two processes to analyze polar and non-polar compounds.[118] A solvent jet is directed onto a sample surface, and the extracted ions are ejected and directed into a heated extended inlet tube on an Agilent 6330 ion trap mass spectrometer where photoionization by vacuum UV (**VUV**) photons takes place. Testing different molecular weight compounds, the group concluded that there are two ionization processes occurring, for small molecules via photoionization and for larger molecules through the heated inlet capillary by the ion evaporation mechanism.

The methods developed for introducing samples directly into the AP inlet aperture are attractive for their simplicity, but in practice, there could be long-term issues of instrument contamination and carryover between successive samples, which are difficult to eliminate similar to other API methods.

Lesson 4:

- A matrix can be a solid or solvent
- Volatility of the matrix is important, especially for producing multiply charged ions
- The new ionization processes allow truly different ion sources such as simply an inlet tube
- We need to obtain a better fundamental understanding of the ionization processes
- SAI developments have seen the greatest expansion, but progress in MAI may change that

5. Fundamentally-Driven Arguments Leading to the Surprising Discovery of Ionization Seemingly Not Possible

In this section we first describe the discovery of the "unthinkable", at the time, using intermediate pressure (*vacuum* ionization conditions) on an intermediate pressure MALDI source. The concept was later transferred to AP (*inlet* ionization) using an API source, but without the need for an inlet tube. We follow briefly with simplifications and showcase initial applications to contrast the previous difficulties. These applied examples were critical in our path forward, finally giving our research legitimacy and propelling the need for serious technical developments to the forefront.

The discovery of LSI was based on considerations of ESI and MALDI mechanistic aspects because of similarities of both: one in terms of charge states (ESI), and the other, in terms of the matrix (MALDI). The use of a laser in TG without voltages gives good results, minimizing the background noise and providing little or no metal cation or matrix adductions, working from AP decreases the fragmentation. The problem-solving principle described by "Occam's razor" or "law of parsimony" offers the insight that the right answer will be found if one pursues the path that "entities should not be multiplied without necessity".[119] In other words, limit your assumptions to a bare minimum. Following this guiding strategy, we looked at what is truly known about ESI and MALDI and used that to explain LSI, MAI, and SAI with the least assumptions.[37] The main difference between MALDI and ESI is the production of singly charged ions by MALDI which limits the analysis of larger molecules with most mass analyzers, *versus* ESI that provides multiply charged ions. Because we were observing multiply charged ions with MALDI-like "tools", the most straight forward explanation is that they are made from charged droplet (molten matrix) or particles and like ESI requires removal of the matrix. The heated inlet tube and collisions with a surface would be means to aid desolvation of the charged gas-phase matrix particles. The use of collision surfaces or obstructions are well-known to enhance ionization in ESI.[120–126] We noted that higher melting and presumably less volatile matrices required higher inlet temperature.

We hypothesize that if some matrices require high inlet tube temperature to produce ions (*e.g.*, CHCA >400 °C)[5,9,37,40] that there may be more volatile compounds that do the same at a lower temperature (**Scheme S1**). This hypothesis guided us in pursuing dedicated studies on potential new solid matrices with *increased* volatility, initially leading us to the discovery of vLSI (note, MALDI matrices were optimized for decades for stability under vacuum[40,44]). A wide-ranging screening study followed in order to discover new matrices and improve the analytical utility and expand to different mass spectrometers.

- *Vacuum* ionization conditions. Nearly at the end of this lengthy (painful) "matrix" study, when a backordered chemical was finally tested for its potential as a matrix for MAI on a Thermo LTQ Velos or vacuum LSI on a Waters

SYNAPT G2, Trimpin and the graduate student, Ellen Inutan, discovered the pinnacle of the astonishing ionization processes. That is, with the matrix 3-NBN, analyte ions were observed in high abundance and multiply charged *without* initiating the laser on a vacuum MALDI source of a SYNAPT G2 at room temperature (**Fig. S15**).[2,3] Even femtomoles of proteins were spontaneously converted to gas-phase ions,[2] and astonishingly, the BSA protein (66 kDa) produced mass spectra with high charge states.[3] Because of the multiply charging, the mass range of the intermediate pressure Q-TOF (SYNAPT G2) was extended. Many other matrices followed this initial discovery (**Scheme S1**) especially after we learned from literature[127] that the 3-NBN compound has a strong triboluminescence behavior. With this breakthrough discovery, analytical useful ionization and a broad range of applications finally followed. A few examples are briefly detailed here.

Using the 3-NBN matrix, reaction products were monitored in positive and negative modes.[11] One of these studies, in collaboration with Dr. Mary Kay Pflum (WSU), provided evidence of the efficiency of *v*MAI where ESI or MALDI were not successful (**Fig. S16**) at least not in such a simple straightforward manner. One key strength is that no acid is required in *v*MAI[128] avoiding undesired hydrolysis hampering some ESI and MALDI applications when standard operation of acid addition is used to improve ionization.[129] Other labile post-translation modifications (**PTM**'s) have been successfully analyzed because no laser is necessary nor is the addition of acids.[7,9,91] vMAI is not only simple but also highly sensitive and applicable to a variety of analytical challenges. One such other example is the differentiation of bacterial samples. The ability to differentiate between strains of the bacteria *Escherichia coli*,[11,130] simply by placing the samples containing the 3-NBN matrix in the intermediate pressure MALDI source without engaging the laser was demonstrated (**Fig. S17**) and provided important proof-of-principle results for subsequent ion source developments by MS^{TM}, detailed below. vMAI using the commercial intermediate pressure MALDI source identified for the first time the unknown antimicrobial peptide (16-mer oncocin) in collaboration with Dr. Christine S. Chow's lab (WSU).[131] Several other types of samples and direct surfaces have been analyzed including tissues.[3,9,10,132]

A simple method to characterize a specific spatially resolved area of a surface is to place a matrix solution on the area of interest and insert the sample into the vacuum of the mass spectrometer. Only the area where the matrix is applied produces ions.[3,9,10,133,134] The ion duration can be several minutes depending on the instrument, matrix, sample preparation, and importantly how much matrix is used in the analysis.[3,11,128] Because of the sensitivity of this approach, we hypothesize

that even smaller depositions of the matrix solution will improve spatial resolution measurements from surfaces without the use of a laser.

Because 3-NBN sublimes[128,135] when in an intermediate pressure source over typically several minutes, in principle, one has this time to acquire several samples on a plate with a single plate introduction to vacuum (*ca.* 2 min). Using improved sample preparation protocols, 24 samples applied to a metal plate as 6 different analytes in 4 repeats produced excellent mass spectra of all components in 4 min, not including the 2 min sample loading.[9,136] No carryover between samples was observed because only if the specific sample is located directly in front of the hexapole ion guide do the gaseous ions transmit to the mass analyzer and become detected (**Fig. S18**). Introducing a sample directly into vacuum, similar to vacuum MALDI but without the use of a laser,[3] provides sensitivity and robustness to carryover and instrument contamination at least comparable to MALDI.[137] The typical mass spectrometer capabilities (IMS, MS/MS) can be readily used without employing the laser.[2,3,133]

Initial experiments demonstrated that the generation of MS images is possible using the vacuum MALDI plate and interface, without the addition of energy (**Fig. S19**). Recently, Clench's group (Sheffield Hallam University) implemented *v*MAI on a modified Q-TOF mass spectrometer fitted with an orthogonal intermediate pressure MALDI source and generated initial results for the generation of images of lipids on brain tissue adding 5% of chloroform in the 3-NBN matrix solution.[138]

Others have also used the vacuum source without engaging the laser. In 2014, Dr. Charles L. Wilkins' group (University of Arkansas) tested *v*MAI on a Bruker Fourier transform ion cyclotron resonance (**FTICR**) mass spectrometer. This was the first time that 3-NBN was used in high vacuum, and the ionization event took about 30 min.[139] Using the laser (355 nm) produced essentially the same results with the exception of speeding up the sample consumption to *ca.* 2 min. This group, in 2018, showed the utility of *v*MAI and 3-NBN to rapidly ionize lipids, demonstrating the potential of *v*MAI-FTMS to differentiate edible oils by their profile of non-polar and polar lipids and its utility for taxonomic identification of bacteria using crude bacteria extracts.[140] Meanwhile, Li's group used *v*MAI[141] to analyze peptides with PTM's, as well as the characterization by CID and HCD on an adapted intermediate pressure MALDI-LTQ-Orbitrap XL.

- *Inlet* ionization conditions. vMAI was first discovered using the intermediate pressure MALDI source on a SYNAPT G2 mass spectrometer. With volatile matrices which sublime when exposed to sub-AP conditions, the 2 minutes to load the sample can be problematic, thus, a faster and more convenient sample introduction to vacuum was sought. The easiest means was the introduction into the inlet aperture

of an API mass spectrometer using *e.g.*, glass plates or pipette tips as sample substrates (**Movie S-02**). This approach provided exceptional simplicity and good sensitivity, without any optimization or application of significant heat (*ca.* 30 °C) and analytically useful results were obtained (**Fig. S20**).[137,142–145] Multiply charged ions are produced similar to ESI so that any mass spectrometer designed for ESI can be used with MAI because the only requirement is the access to the sub-AP of the mass spectrometer and an inexpensive matrix such as 3-NBN. With this matrix, an inlet tube is unnecessary and, in fact, added heat typically hampers analyte ion abundance.[128,145] The condition required for ionization to occur is that the matrix must sublime/evaporate.[2,37] The *v*MAI matrices are so volatile that many of them disappear from a glass plate held at room temperature over a 3 to 24 h period, as was monitored by microscopy.[128]

The 3-NBN matrix is rather selective in that many common contaminants observed with ESI are not efficiently ionized with 3-NBN, partly because 3-NBN discriminates strongly against ionization by metal cation addition.[5,8,128] Of course, this is often an advantage, specifically for biological MS as is shown in **Fig. S21** of proteins in collaboration with Drs. Young-Hoon Ahn (WSU) and Ken Mackie. The proteins methyltransferase (SMYD2), with an expected molecular weight of 49,668 Da (experimental determined 49,844 Da) and EBOV VP40 an Ebola virus protein with a molecular weight of "*ca.* 35,000 Da" (experimentally determined 35127 Da), were readily ionized from buffer solutions diluted 4:1 with water and mixed with 3-NBN. The cone temperature was set to 30 °C. For the deconvolution we used MagTran,[146] a Freeware deconvolution software for ESI mass spectral data. Both samples were analyzed using a pipet tip, therefore there is somewhat of a concern for contamination just like with any other direct injection method used in MS.[10,137,145] While 3-NBN discriminates against metal adduction, other MAI matrices ionize analyte by metal cation adduction. The selectivity as well as ionization is matrix dependent.[8,128,147,148]

Sensitivity equivalent to nanoESI has been achieved using the 3-NBN matrix by inserting the matrix:analyte sample directly into the inlet of an API mass spectrometer.[149] In collaboration with Chow's lab, a synthesized heptapeptide on TentaGel® resin beads of 75 μm size was photocleaved and promptly analyzed by MAI-MS and MS/MS using a variety of considerations such as charge states as well as CID and ETD on two different mass spectrometers. The peptide material was collected and used for the measurements shown in **Fig. S22**. A slight preference for peptide sequencing using ETD and ProSight Lite[150] for data interpretation is observed.

The most commonly used matrices in MS to date are depicted in **Scheme S1.** Binary matrices have been recently discovered which provide in-

creased sensitivity and may provide tunable selectivity and desirable synergistic properties.[8,10,14–16,151,152] This area of research is in its infancy but holds considerable promise for further improving the MAI and *v*MAI processes. A large number of compounds are found to act as matrices, including solvents.[2,47,51,54,87,128,147] These solid and liquid matrices and matrix combination can be used with or without employing a laser, high or low voltages, at various temperatures (above and below room temperature), and gas flow, but they are typically not necessary and can hamper the production of ions, especially those that are multiply charged.[5,8,10,37,145] While a laser is not required for ionization,[2,3,22,133,137,142,144] lasers have been used to achieve high spatial resolution, as in LSI.[23,26,30,41,42,44,62,64,153–155]

A variety of different matrices showed improvements for different analyte types. Synthetic carbohydrate-lipid conjugates were successfully ionized in the negative ion mode using 2-bromo-2-nitropropane-1,3-diol (bronopol) matrix (**Scheme S1**). The ionization occurs in the negative ion mode through deprotonation (**Fig. S23**).[147] Importantly, using traditional ESI or MALDI analyses failed. This was the first example in which these complicated demanding structures have been detected by MS. With this, Dr. Zhongwu Guo's group (WSU), for the first time was able to obtain molecular ions of what they synthesized,[156] and seized the opportunity for improving their synthetic and clean up procedures.[147]

The MAI matrix 1,2-dicyanobenzene (**1,2-DCB**) (**Scheme S1**) improves the negative mode detection of gangliosides and cardiolipins. The analysis of gangliosides directly from tissue sections in the negative ion mode was performed using a glass plate containing 10 μm thick mouse brain coated with 1,2-DCB on the Z-spray source of a Waters SYNAPT G2. IMS separation was used to help further separate and identify the gangliosides by inclusion of drift times (**Fig. S24**).[157] These acquisition conditions were also applicable to the analysis of mitochondrial membrane extracts. Cardiolipins are abundant in mitochondria. The experimental setup consisted of the direct extraction of these compounds from the liver, brain and heart of rats. The MAI analyses were performed with a pipet tip by bringing the tip close enough to the inlet, or touching against the inlet of an ESI source, typically held at 50 °C, on a Waters SYNAPT G2 instrument. These quick experiments were performed by a visiting graduate student from the medical school laboratory of Drs. Thomas H. Sanderson (WSU) and Karin Przyklenk (WSU) and suggests potential use for clinical applications (**Fig. S25**).[158] In terms of quantification using MAI, Halverson's group (University of Oslo) demonstrated an initial, but very promising utility of MAI-MS of proteomic applications using 3-NBN as matrix.[159]

In order to automate MAI analyses, sequential samples held at AP using an array of pipet tips was

demonstrated on two different mass spectrometers, a linear ion trap LTQ Velos and on a Waters SYNAPT G2. A programmable X,Y,Z-stage was used to align and move samples at AP to the vacuum orifice entrance. Eight samples consisting of small and large compounds were analyzed in 1.22 min.[145] The same two instruments and MAI were subjected to a reproducibility study, using six drugs, two matrices, and on different days. The reproducibility is improved with improvements in matrix:analyte sample introduction into sub-AP of the mass spectrometer and quantification was demonstrated to be comparable with ESI and MALDI using internal standards.[144] In collaboration with Dr. Srinivas B. Narayan (Detroit Medical Center), the urine of a newborn was identified to have been exposed to drugs from the mother during pregnancy at levels similar to those determined by ESI. With MAI, one can manipulate the cross-contamination issues using different approaches, choosing a more volatile matrix, taking advantage of the airflow between samples, or by increasing the inlet temperature.[7,128,145,160,161]

Early work showed the applicability of "dissolved MAI" (**Fig. S26**).[37] This led to another approach that we used in our laboratory in which a premixed matrix:analyte solution is continuously infused into the inlet of the mass spectrometer prolonging the ion abundance.[40,91,162] The idea of mixing solution with the best performing matrix (3-NBN) was also shown by Dr. Marcos N. Eberlin's group (University of Campinas) in 2015 who reported 3-NBN as a solvent dopant to increase the sensitivity in the method called easy ambient sonic-spray ionization (**EASI-MS**).[163]

The AP approach of MAI appears applicable on a wide variety of instruments as has been shown in proof-of-principle applications through the years *e.g.*, various Thermo, Waters, Advion, Bruker, SCIEX, Agilent, and homebuilt IMS-MS instruments (**Fig. S27** and **Movie S-03**).[11,16,164]

For the direct analysis of tissue sections provided by Dr. Ken Mackie's lab we demonstrated improvements using a laser and a binary matrix combination (97% 3-NBN: 3% 2,5-DHAP) that allows enough stability on the tissue, enough absorption at the laser wavelength (337 nm, N_2 laser) and sufficient volatility in the gas phase, at least in parts associated with sufficient airflow using a slightly modified skimmer inlet[155] on an SYNAPT G2. A protein with an average MW of 14,135 Da was detected (**Fig. S28**), along with lipids. Operating from AP, 3-NBN (which has absorption at 266 nm[64] matrix can be used with a laser and a variety of different laser wavelengths, as is the case with other MAI matrices in conjunction with sources and modifications.[139,155] The use of a laser can offer advantages of spatial resolution and speed, especially with the laser in TG mode, however, laserless conditions can be employed in which a sufficiently volatile matrix can be used and ions/charged particles are flow entrapped, especially when low heat is applied to the inlet tube or the source block.[8,52]

Other research groups have modified the MAI approach, *e.g.,* Murray's lab developed a variation of MAI using an electrically actuated pulse valve to deliver the matrix:sample into the inlet of the mass spectrometer. This group discussed possible applications for imaging using the pulse to give spatial resolution.[165] In 2019, the same group presented another modification of MAI[166] this time using a piezoelectric cantilever with a needle tip attached that was used to strike the surface of the matrix:analyte set on 25-mm-thick aluminum foil. Same year, Murray's lab implemented the use of silica nanoparticles and the 2-NPG matrix,[77] this co-matrix was added on the top of frozen mouse brain tissue to detect phospholipids and gangliosides. No proteins or peptides were detected directly from the tissue. This analysis was performed under a commercial high vacuum on a MALDI TOF mass spectrometer.

In an interesting study by Li's group, three ionization methods, MALDI, LSI, and MAI,[167] were compared using an AP-MALDI Ultra High-Resolution source (MassTech) coupled to a Q-Exactive HF Hybrid Quadrupole-Orbitrap mass spectrometer and compared to an intermediate pressure MALDI-LTQ-Orbitrap XL. This study concluded that LSI and MAI expand the *m*/*z* range and improve the intentional fragmentation efficiency using ETD, because it provides the beneficial multiply charged ions in comparison with the singly charged ions from MALDI.

In another recent report the tip of a wire containing crystallized matrix:analyte was introduced through the interface capillary of the mass spectrometer into the sub-AP of an ion funnel. In this vacuum region, the matrix containing the analyte is removed by sublimation from the wire and the formed gaseous charged clusters and ions are collected by the ion funnel and transferred to the mass analyzer. The objective of this work was to study the photophysical properties and photochemical activity of fullerene-dyad to generate reactive oxygen species (**ROS**).[168]

IMS-MS has now become 'mainstream' technology of gas-phase separation and structural characterization offering unique opportunities to enhance molecular analyses and imaging where LC cannot be applied.[169] Combining *v*MAI surface analysis with high resolution MS, IMS, and MS/MS provides exceptionally powerful tools common with API but not MALDI mass spectrometers. Recently, together with Dr. Larsen at DuPont, we demonstrated the utility of the 'pictorial snapshot' using *v*MAI-IMS-MS as a means of observing small changes in complex polymer systems.[8,170] We hypothesize that this approach can also be valuable in detecting small differences in the chemical composition of surfaces. In another example, the ability of *v*MAI in combination with IMS-MS to detect even small changes in the conformations of the protein lysozyme under reducing agent conditions was demonstrated (**Fig. S29**).[174] More unfolding studies were performed using a

MAI-IMS-MS approach. Based on the 2-dimensional IMS-MS plots, these unfolding processes are rapidly monitored (**Fig**. **S30A**) and, unlike ESI, without concerns for capillary clogging.[175,176] We used the Orbitrap Fusion in Dr. Paul Stemmer's lab (WSU) to decipher the differences of two mass units as a result of cleavage of 4 disulfide bridges in lysozyme (**Fig**. **S30B**). Structural similarities and differences have been reported using the well-studied model system of ubiquitin, on commercial and homebuilt IMS-MS mass spectrometers based on MAI, SAI, and ESI methods.[43] As an interesting note, on the homebuilt IMS-MS instrument in Dr. David E. Clemmer's lab (Indiana University), the ion abundance was noticeably improved by adding the matrix containing proteins solution directly into the inlet of the mass spectrometers instead of introducing the dried matrix:analyte crystals. Instead of observing the elongated ion duration, a burst of analyte ions was recorded after *ca.* 15 to 20 seconds after introduction to the vacuum. We hypothesize that the sample solution 'collects' on the 'jet disrupter' and that, after the solvent evaporates and conditions become "right", the ubiquitin ions are "explosively" produced under sub-AP conditions. More details on aspects of "sublimation boiling" are discussed in ref. [15]. In the area of IMS using the new ionization processes there is much work to be accomplished in the future.

Thus, for a breadth of materials characterization, an increase in ionization sensitivity, selectivity, and range of compounds detected in a direct analysis approach not only enhances monolayer surface analyses, but also bulk analyses where chemical complexity is combined with the need for direct analyses without use of liquid separation technologies. These include targeted high throughput analyses, and the need for rapid answers, common challenges in the industry and in the clinical area. The use of a "vacuum MALDI source without employing a laser", was demonstrated as proof-of-principle but in reality, is not a practical approach.

MALDI, which is most commonly used with MALDI-TOF,[81–83,177–181] suffers from several disadvantages relative to *v*MAI-MS including higher low-mass chemical noise, frequently harsher ionization resulting in significantly more fragmentation, chemical background, and lower mass resolution and mass accuracy on MALDI-TOF *versus* many ESI-based mass spectrometers.[129] Distinguishing fragment ions from molecular ions can be difficult when they occur during ionization (in-source fragmentation) or during sample preparation (hydrolysis). Further, fewer labs have MALDI-TOF than ESI mass spectrometers, for which only the latter can be readily retrofitted with more than one ionization source. As depicted in **Scheme 1**, API mass spectrometers can be retrofitted for use with the new ionization processes

with minor instrument modifications, or more effectively through inexpensive source replacements. An advantage of MALDI and LSI is the inherent spatial resolution afforded by laser ablation.[18,23,26,28–30,182,183] Although LSI can be used, under certain conditions, with a laser together with a typical "MALDI matrix" (*e.g.*, 2,5-DHB, 2,5-DHAP, even CHCA)[22,26,40,47,63] the acquisition conditions are more gentle and the ionization processes more similar to that of ESI leading to minor chemical background and the absence of fragmentation of multiply charged ions.[22,25,91]

General advantages of *v*MAI-MS for molecular chemical surface analyses include the simplicity of the method which translates into lower cost, its ability to operate on common mass spectrometers used with ESI or APCI including ultra-high resolution and mass accuracy instruments, as well as the ability to produce multiply charged ions for improved mass-selected fragmentation and IMS separations, and the softness of the ionization process. However, analyte ionization can persist after introduction to the inlet aperture of the ESI mass spectrometer, which is a 'curse' using the traditional ESI source, but also a 'blessing' as described under state-of-the-art in section 1 for the developments of ultra-fast analyses with exceptional robustness. Thus, the disadvantage of MAI using the inlet of an ESI mass spectrometer as an ion source is associated with high throughput analyses because of the length of ion duration for each 'injection', as well as potential carryover between samples with high concentration of analyte.[145] One way to obtain some meaningful control over the ion duration is with increased inlet temperature[5,128,137] as was also briefly described above. Further, the ionization process has been shown to require matrix sublimation which, intrinsically, also eliminates matrix-related instrument contamination.[7,10] In other words, sample introduction through the AP inlet was insufficiently robust and too slow for some applications involving the desire or need for ultra-high speed. Thus, not only is the commercial vacuum MALDI source not ideally suited, but also the commercial ESI and APCI sources are limited in their utility, despite their successful initial applications using the new ionization processes developed into new methods for use in MS. To get to a stage of advanced applications, dedicated sources and platforms needed to be developed. Before we describe these instrument efforts, we dedicate a section of mechanistic arguments relative to the exceptionally interesting and important function of a "matrix" that empowers large nonvolatile compounds such as fragile proteins to be analyzed using either a solid, solvent matrix, or combinations without the need of any of the known tools in MS such as high voltages, laser(s), or even heat being applied so long as the source geometry is "correct" for the new ionization processes.

Lesson 5:

- "Life is infinitely stranger than anything which the mind of man could invent", by Sir Arthur Conan Doyle.[184]

- The ion source can be as simple as a hole to the vacuum of the mass spectrometer, but there is room for improvements
- With *vacuum* ionization it is more difficult to create meaningful improvements, but it is potentially more important because of robustness and sensitivity
- Intriguing new applications possible (when the technology is developed properly)

6. Mechanistic Arguments Relative to Matrices, Pressure Regions, and Charge States

The previous sections have covered the fundamentals and mechanistic arguments obtained from studying these new ionization processes over the last decade. Our research has been guided through successive fundamentally driven-research questions for which we have published a number of papers.[5,7–10,35,37] It should be noted that the most recent of these fundamental papers is included in this Biemann Special Issue honoring the discovery and developments of the new ionization processes.[15] The following section is devoted to the fundamentals related to "the matrix" for the ionization of small and large nonvolatile molecules, and characteristics of a 'good' matrix. The discussion of the results is primarily based on the charge states obtained under different pressure conditions. Q-TOF and TOF mass spectrometers at different pressure regimes, without and with a laser to assess their ability assisting in analyte ionization by potentially adding the required energy under high-vacuum conditions. A more detailed discussion can be found in the Supplemental Information of this *Perspective.*

Solvents and even MALDI matrices (**Scheme S1**) produce analyte ions with charge states similar to ESI when traversing an inlet tube.[5,7–10,37,54] An important development was the discovery of matrices which produce ions simply by exposure to sub-AP conditions at room temperature without need of an inlet tube. Thus, the first discovered matrix, 3-NBN, produces abundant analyte ions when introduced into a room temperature inlet tube, a skimmer separating AP from vacuum, or directly into vacuum on a substrate.[2,3] Because 3-NBN sublimes under vacuum, it is pumped from lens elements and is thus self-cleaning.[7] However, the importance of a subliming matrix goes beyond self-cleaning in that the sublimation process appears to drive ionization by expelling matrix particles into the gas phase in a process which may be akin to boiling in liquids.[15] The expelled particles need to acquire a charge, and while statistical charging will occur, a process such as that responsible for charge separation in triboluminescence seems to be necessary to achieve the high charge states observed. Indeed, several of the more successful MAI matrices discovered are known to be triboluminescent (**Scheme S1**)[2,3,5,9,127,133,185,186] and have been shown to sublime even at room temperature and pressure (**Fig. S31** and **Movie S-02**).[128] The use of matrices, of course, is not novel. In field desorption (**FD**) the use of benzonitrile is fundamental for the preparation of carbon dendrites in the emitters,[187,188] and even though plasma desorption (**PD**),[189] by itself does not need

a matrix, it was improved with the use of nitrocellulose and glutathione matrices.[190,191] Fast atom bombardment (**FAB**) introduced the use of glycerol as a liquid matrix.[192–194] Later, with the development of MALDI the use of the liquid matrix glycerol was successful for ionizing proteins.[195] The use of nicotinic acid, a small molecular weight solid phase compound as matrix was used to ionize larger proteins.[196] In the case of ESI, the analyte needs to be dissolved into a liquid matrix to be dispersed in a fine aerosol.[197] In the end, the matrix plays a critical role in the ionization processes (**Scheme S2**).

Experiments suggest that, similar to desolvation in ESI, the charged matrix particles in MAI must 'desolvate' by a sublimation or evaporation process. We find evidence that for some less volatile matrices or conditions, loss of matrix is not complete even at the TriWave ion mobility region of the SYNAPT G2(S) mass spectrometers.[5,7,9] This suggests that improved 'desolvation' conditions or more volatile matrices could improve sensitivity. Our laboratory has discovered a number of other matrices (in the order of 50 self-ionizing MAI matrices[2,11,15,128] *versus* MAI matrices at high heat >200,[22,37,40] **Scheme S3**) that spontaneously produce analyte ions when associated with analyte, dried, and exposed to sub-AP conditions without need of a laser, ion bombardment, voltage, or heat.[5,9] Similar to MALDI, each matrix has different compound selectivity as well as preferences relative to positive or negative ions. For the 3-NBN matrix using the intermediate pressure source on a Waters Q-TOF SYNAPT G2, BSA ions were detected with up to +37 charges (vMAI),[3] and the matrix:analyte sample addition from AP using a modified skimmer cone orifice on the same instrument produced +39 charges (MAI) with the inlet temperature set at 30 °C.[9] In comparison, using the matrix 2-NPG under high-vacuum on a Bruker MALDI-TOF with the use of a laser, +14 charges are detected (*v*MAI).[64] On the other hand, using the 2-NPG matrix with the use of a heated (200 °C) inlet tube of a Thermo LTQ Velos instrument, +67 charges are observed (MAI).[79]

In MALDI, clusters have been discussed;[37,71,198–203] as an alternative to the photoionization mechanism.[204–208] We, therefore, hypothesize that the successes of MAI matrix compounds, which also represent LSI and SAI matrices, are associated with three key steps for ionization to occur (**Scheme S3**): (**1**) *sublimation/evaporation* which assists in expelling small matrix particles from the surface into the gas phase,[15,37,40] (**2**) a *triboluminescence-like process* of charge separation which results in the expelled particles being positively and negatively charged,[2,128] and (**3**) *sublimation/evaporation* of the matrix from the charged gas-phase particles to release the bare analyte ions.[5,9,35,37] Directly related to this argument, but separate from the ionization process itself may be the necessity for analyte molecules to be solvated by residual solvent or the matrix in order to

be able to produce gas-phase ions.[8] Recent laserless results using a binary matrix combination of 3-NBN/CHCA demonstrated improvements in ionization of lysozyme, which may be explained by CHCA aiding in keeping the protein solvated.[15] The three ionization parameters are more restrictive under vacuum relative to the high gas flow with inlet introduction resulting in fewer 'good' matrices for *v*MAI.[209] As is shown in **Table 1**, the new matrices, selected for their known ability to triboluminesce[127] and hypothesized volatility, were subjected to a very intense study from AP to high vacuum mass spectrometers, with and without the use of a laser, concluding that triboluminescence and volatility together are key for these new ionization processes, similar to what was seen in previously reported work.[5,7–11,15,16,209] In other words, fewer matrices are still providing the matrix assistance for either forming multiply or singly charged ions of the analytes studied when decreasing the pressure from atmospheric, to intermediate, to high vacuum. In a study also presented in this Special Issue,[15] we compared the ability to produce ions for three different matrices. Coumarin presented the best performance for ion production, in comparison with acenaphthene. On the other hand, acenaphthene presented an interesting behavior, producing a radical cation for the specific analyte case phenothiazine. In McEwen *et al*[15] we described the mechanistic arguments around these new ionization processes. For this reason, these results will not be explicitly covered in this *Perspective*.

More systematic studies under high vacuum TOF conditions are needed to elucidate and more completely understand the capabilities of matrices, especially as a function of the pressure (vacuum) as well as considering temperature arguments. Most of high vacuum TOF measurements described in the following studies were performed in our collaborator's lab, Dr. Chi-Kung Ni (Academia Sinica, Taiwan), and additional measurements at the facility at University of Toledo, at DuPont (Dr. Larsen), Bundesanstalt für Materialforschung und –prüfung (Dr. Weidner), and WSU.[7,8,47,52,170] For a more detailed description of these fundamental studies, refer to the discussion in SI.

Table 1. *Compounds tested under different conditions. The * symbol indicates compounds with known triboluminescence characteristics.[210] The # symbol represents the binary matrix combination (for results see e.g., ref. 8); these two compounds used individually performed significantly less well.[128] √√√ Indicates high abundance of multiply charged ions of the analyte studied without the use of a laser, X no analyte ions without a laser, 355 nm is the laser wavelength used at relative low laser fluence for ach compound. For results obtained on high vacuum TOF mass spectrometer at various wavelengths refer to ref. [64] and* ***Fig. S32****. Melting points (m.p.) were obtained from publically available commercial sources, MW stands for molecular weights, UV stands for the absorption characteristics acquired (****Fig. S33****), AP stands for atmospheric pressure, homebuilt stands for the vacuum-probe source,[12] IP refers to intermediate pressure, commercial refers to commercial MALDI source without initiating the laser.[2]*

Matrix	mp (°C)	MW (Da)	UV (nm)	AP-MAI	Homebuilt IP-MAI	Commercial IP-MAI
Coumarin*	68-76	146.14	266	√√√	√√√	√√√
6-Methylcoumarin	73-76	160.17	266	√√	√	X
1,3-Diphenyl-1,3-propanedione*	77-79	224.25	355	√	X	X $laser_{355}$
Avobenzone	83.5	310.39	355	√√	X	X $laser_{355}$
1,3-Dinitrobenzene	84-86	168.11	266	√√	√√	√√
Propham*	90	179.22	266	√√√	√√√	√√√
Acenaphthene*	90-94	154.21	266	√√	√√	√√
1,3-Bis-(3,5-dimethyl-phenyl)-propane-1,3-dione	96	280.37	355	√√	X	X $laser_{355}$
9,10-Dihydroanthracene*	103-107	180.25	266	√√	X	√
Resorcinol*	109-112	110.11	266	√	√√	√√
Phthalic anhydride*	131-134	148.12	266	√	√√	√√
1:1 Binary 1,3-DNB and 1,3-DCB #	---	---	266	√√√	√√√	√√√

To appreciate the changes based on vacuum conditions, we first describe general trends that are summarized in **Scheme S3** relative to matrices used without and with a laser and the more challenging formation of ions, especially multiply charged, at colder conditions. An interesting observation is that the degree of multiply charged ions from AP may vary,[9,10,37,43] however, the formation of only singly charged ions under voltage free conditions, with or without a laser,[22,26] has not been observed using the new ionization processes. From intermediate pressure, a limited number of matrices under certain acquisition conditions allowed the observation of solely singly versus multiply charged ions from the same matrix:analyte sample spot based on the energy added.[10,37] As examples, the well-functioning MAI and LSI matrices (2,5-DHAP and 2-NPG)[5,9,44,62,64,65,128] produced multiply charged ions from AP and intermediate pressure. In the unique case of 2,5-DHAP solely singly charged ions can be formed at will from the same matrix:analyte spot when high energy is imparted (laser, voltages, gases).[37] More recently the matrix 3-NBN was shown to produce singly charged ions from intermediate pressure,

even without employing a laser.[10] Under high vacuum, only singly charged ions are formed with 2,5-DHB and 2,5-DHAP.[52,64] Ions for bovine insulin are only observed when there is a "match" between the matrix absorption and the laser wavelength used.[64] Contrary, using a laser at the wavelength of absorption of the 3-NBN matrix (266 nm) initiates analyte ionization where the ions observed are *multiply* charged on a high vacuum TOF mass spectrometer. This could suggest that the laser adds the additional 'heat' (thermal energy) necessary for desolvation under these high vacuum, cold, conditions. However, high vacuum studies applying well-developed methods used to understand the MALDI mechanism showed that with increasing laser fluence added to the MAI matrix, 3-NBN, a substantially increasing degree of singly charged ions are formed.[64] Comparisons were made with AP measurements that did not show this charge state dependence. These studies confirmed previous results from us and others[7,9,128] and together with *ab initio* calculations[52] concluded that the MAI 3-NBN matrix does not act like a MALDI matrix even on a high vacuum TOF mass spectrometer. An example of bovine insulin using 3-NBN is shown in **Fig. S32**. While increasing the laser fluence shows a decrease in multiply charged ions, an increase is observed for singly charged ions.

We also analyzed BSA, 66 kDa. **Fig. S34** describes the behavior when the laser fluence is increased using different matrices. 3-NBN failed to produce BSA ions so far from high vacuum, contrary to the same measurements previously obtained for bovine insulin (5.7 kDa).[55] It appears that those MALDI matrices that form an increasing degree of multiply charged ions exhibit a smaller total ion intensity increase (*e.g.*, CHCA relative to *e.g.,* 2,5-DHB). More *vacuum* ionization studies will be required to solidify these trends, similar to the in-depth studies performed by us and others on *inlet* ionization and variations thereof, detailed above.

Absorption measurements of MAI matrices were performed (**Fig. S33**) and analyzed with AP, vMAI, vLSI from AP, intermediate pressure, and high vacuum. The most interesting matrices where shipped to our collaborator, Dr. Ni, for further studies. Matrices that perform well from higher pressure (AP) no longer work well at intermediate pressure and fail at high vacuum or produce singly charged ions. On MALDI-TOF mass spectrometers, vMAI has not been observed. Ionization of bovine insulin at high vacuum involved use of a laser, and using various wavelengths depending on the matrix absorption. The matrices studied at each absorption showed that even relatively volatile MAI matrices with known triboluminescence no longer formed analyte ions from high vacuum (**Table S2**, **Fig. S35** and **S36**). Overall, the following two matrix results stand out: methyl-5-nitro-2-furoate and coumarin. Even one of the most volatile matrices methyl-5-nitro-2-fu-

roate[128] no longer produced multiply charged analyte ions, but only singly charged ions from bovine insulin (**Fig. S37**), similar to other MAI matrices studied at high vacuum (e.g., 2,5-DHAP and 2-NPG).[64,209] Coumarin is also a rather volatile matrix, and is also a known triboluminescent compound.[186,211] The coumarin matrix enabled the formation of the doubly charged bovine insulin ions but not to the degree of the 3-NBN matrix. We hypothesized that the differences might be related to the relative ability to sublime/charge separate, hence we began further temperature studies.[2,51,128]

Initial results with a SYNAPT G2 in which the plate was cooled to -80 °C and promptly introduced to the intermediate pressure of the mass spectrometer showed curious results. Using the matrices 2-NBN and 3-NBN, doubly charged ions were produced when the laser was fired, but after two minutes singly charged ions were observed. The matrix 1,2-nitrophenol, known to only work at high inlet tube temperatures,[44] did not produce ions under these cold vacuum conditions using a laser. The effect of temperature on initiating the sublimation process was also examined, as seen in the total ion chronograms (**TIC**'s) (**Fig. S38**) and in a subsequent frozen condition with the use of a laser (**Fig. S39**). Preliminary data on a *ca.* -80 °C cooling system on a high vacuum TOF mass spectrometer, similar to previous work[212] in Dr. Chi-Kung Ni's lab, produced singly charged ions for insulin using laser ablation with the 3-NBN matrix. However, at room temperature multiply charged ions were observed at high vacuum.[209] Scheme **S3** summarizes connections between temperature, pressure, and the number of matrices which are operational at various pressures and their propensity to form singly or multiply charged ions. These are preliminary results, and many more dedicated studies will be needed to understand the underlying fundamental reasons for the vacuum and temperature dependence for the respective matrix molecules.

We have used RAMAN spectroscopy to study the potential presence of molecular interactions between the matrix:analyte.[7] In one study, we analyzed the matrix 3-NBN, the analyte angiotensin II, and the matrix:analyte combination. One of the challenges in using what is considered the 'normal' sample preparation and extract meaningful data is that the analyte is in low abundance relative to the presence of the matrix (*ca.* 1:100,000 is common). Preliminary results, however, suggest there is an interaction between the matrix and analyte, even though other studies have indicated analyte incorporation into the matrix is not necessary.[8,52] A more systematic study needs to be performed in order to conclude what the implications of these interactions in terms of sublimation and ion generation might be. Single crystal X-ray results show that the 2-NPG matrix contains water molecules in its crystal structure,[8] whereas this was not the case for 3-NBN.[213] We hypothesize that obtaining a better understanding of the pro-

cess that leads to improved results with some binary matrices[14–16,37,65,151] will allow us to better tailor the solvation and ionization processes to achieve desired results.

Relative to the molecular composition of the matrix, we recently published a fundamental study that focused on the importance of water (and other protic solvents) being present during vMAI sample preparation, especially for ionization of peptides and proteins.[8] Matrix combinations have been found which produce different selectivity than the individual compounds used as matrices and some provide as much as two orders of magnitude improved sensitivity *versus* the individual matrices. With relatively little research effort in binary matrix combinations, we recently produced a number of matrix combinations[14–16,151] that the students now call 'super matrices' (*e.g.*, 1:1, *v*/*v* of 1,3-DCB and 1,3-DNB, and 2:1 *v*/*v* 3-NBN and CHCA) (**Table 1**).[10] The combination of matrix compounds produces this synergy.[16,151] We hypothesize that by gaining an understanding of how this synergy works and combining that with our basic understanding of matrix selectivity, we will be able to tune matrix combinations and improve sensitivity. Such an outcome will be especially useful for surface analysis such as tissues, films, and monolayers.[16,170,214]

One constant from the beginning of MAI was the recognition that sample preparation and possibly even humidity matters. However, to date, no study of sample preparation has yielded a specific protocol to achieve the best possible results, although several papers have dealt with specific opportunities related to sample preparation.[2,3,40,65,128,215] In one example, screening through *ca*. two dozen MAI matrices using solutions with different pH values showed no improvements relative to the pH obtained after the compounds are dissolved, and typically a noticeable decrease instead.[128] Interestingly enough, analyte in basic solutions were frequently still detected in the positive mode and with charge states similar to ESI.[40,128] Several students have suggested that crystallization is affected by laboratory conditions, especially humidity, in addition to the solvent composition (with student comments such as 'films are bad', 'crystals are good', small crystals for AP and larger crystals for vacuum conditions are observational reports). We hypothesize that a systematic study of sample preparation protocols, especially with additives[215] and under stable atmospheric conditions, will lead to increased sensitivity and ionization stability.

As described above, for several of our collaborators the main interest is the identification of materials directly from surfaces. Successful molecular analysis by MS requires removal of molecules from the surface as gas-phase ions. Ideally, this occurs under mild conditions that do not alter the molecular structures as they exist on the surface. With vMAI, as with MALDI, the solvent used to dissolve the matrix, and likely the matrix itself, are primarily responsible for dissolution

(solvation) of the adsorbed surface layer. Thus, both the solvent and the matrix are potentially important in enhancing the sensitivity of surface analysis simply by the efficient dissolution of the surface layer. We, therefore, instituted a brief search for more effective solvents for analysis directly from surfaces, with the realization that dissolution is likely compound specific. Both water (ε 80.4) and methanol (ε 35) have significant dielectric constants at room temperature, and these solvents seem necessary for high sensitivity MAI. We, therefore, looked at formamide (ε 111).[216] It will be of interest to determine if formamide or similar dielectric constant material plays a large role in surface analysis. Recent studies identified formamide as an alternative solvent for vMAI[16] (**Fig. S40**) suggesting its potential use in surface analysis of polar and ionic compounds. Formamide may enhance the analysis not only through improved desolvation of compounds distributed on a surface but also for its charge separation properties.[216,217]

Molecular modeling can provide insights into chemical processes once initial research has been accomplished. Using the density functional theory (**DFT**) calculations in collaboration with Dr. Schlegel (WSU), for water as a matrix in SAI, the ionization of small molecules containing electron-donating groups followed closely, but not perfectly, the pk_a.[218] Similar studies for MAI were less conclusive relative to the performance dependence to the analyte basicity and the MAI matrix 3-NBN. The analyses of larger molecules using SAI or MAI cannot be modelled by DFT. However, the experimental results for the simple case of MAI with the 3-NBN matrix dissolved in water and acetonitrile or acetone support the relationship of basicity (isoelectric point) of the proteins having critical influence, and less so that of the molecular weight, at least for the positive mode measurements (**Table S3**) as was shown in collaboration with Drs. Ahn and Mackie.[219] Ni's group used theoretical calculations (molecular geometry and reaction barrier) and concluded that the mechanism of ionization using 3-NBN as a matrix and the 266 nm laser which forms gaseous highly charged ions of bovine insulin under high vacuum conditions,[64] is different than that of MALDI as compared to known MALDI matrix calculations.[64] Without modeling, we predicted in a 2012 publication that a "MAIV" process might be possible,[37] despite 20+ years of literature contradicting our prediction. Following through on our hypothesis led to MAI results which have greatly exceeded our expectations and that of the community as evidenced by honoring the subject matter with the Biemann Medal.

Lesson 6:

- How matrices function is complex, but important to understand
- There are significant differences between AP, intermediate pressure, and *vacuum* ionization using the same matrix and sometimes the same mass spectrometer Fewer compounds act as MAI matrices as the pressure is decreased.

- Matrix absorption and volatility are a necessary but not sufficient condition for forming the multiply charged ions at high vacuum by laser ablation. A process like triboluminescence may also be helpful
- Less volatile matrices require higher energy input, and higher energy is associated with singly charged ions.

7. Advanced Technologies and Enhanced Applications using Inlet and Vacuum Ionization

To take full advantage of the new ionization processes, we recognized that dedicated platforms and sources will need to be developed, in contrast to the simpler developments described above. The fundamental studies depicted throughout this text guided and enabled the successful platform and source designs. Applications, however, are key to demonstrating the utility of the new ionization technologies.

- *Inlet ionization – Ionique platforms*:

The development of the manual and the automated Ionique platforms are making the new ionization methods significantly simpler and more reliable than using the initial approaches of, *e.g.,* pipette tips.[55,142,145] Both Ionique platforms by MS^{TM} support multiple ionization methods, including MAI, SAI, and ESI.[9,10,220] Initial applications to the clinical area were tested by analyzing raw urine samples obtained from the Detroit Medical Center. Samples were placed in microtiter wells and analyzed using MAI (**Fig. S41**). Forensics and portable MS applications have also been demonstrated.[84,132,164,221]

The manual Ionique platform allows MAI by inserting solid matrix:analyte sample on a 1 μL syringe needle into the inlet tube of a mass spectrometer. Typically, only 0.1 – 0.2 μL of sample is dried on the tip of the syringe capillary, and because the matrix sublimes and the sample is generally present in less than a picomole, inlet contamination is no worse than ESI. SAI can be performed by inserting an analyte solution into the heated inlet tube, and usually is performed with 1 μL, or less, expelled into the inlet. With ESI, again 1 μL is expelled from the syringe capillary, but about 1 cm in front of the inlet and with a voltage applied to the metal syringe capillary needle. Each method has advantages and disadvantages which are clearly seen with complex mixtures. Interestingly, with biological samples, ESI is prone to ionize by metal cation adduction, whereas MAI with 3-NBN does not. The advantage for ESI is that compounds which ionize only by metal cation adduction are observed in ESI and not MAI. However, the downside is that some compounds are ionized by proton, sodium, and potassium cation adduction in ESI, while in MAI only by protonation, greatly simplifying the mass spectra. With bacterial samples acquired by ESI and MAI, protein multiply charged ions were frequently obvious in the mass spectrum and only protonated, whereas with ESI without addition of formic acid, these ions had multiple cation adduction and were frequently of low abundance relative to low-mass

ions. Initial results with the manual platform using MAI showed that with a reproducible sample introduction, quantification with the use of internal standards was equivalent to ESI, but faster. Finally, the manual platform can be interfaced with LC using ESI or SAI[85] and other combinations which require a pumping system for continuous or discreet flow applications.[89] In one example, an undergraduate student analyzed 25 standards of carbohydrates from our collaborator, Dr. Crich (University of Georgia). The ionization method used was SAI and the analyses were performed on the SYNAPT G2S with the modified heated inlet at 250 °C. **Fig. S42** shows a comparison between SAI and ESI. The carbohydrate analysis is feasible using the new ionization processes. The applicability to lanthanide analyses is shown using the heated MS™ inlet tube on the SYNAPT G2S (**Fig. S43, Table S4(a)** and **S4(b)**). The approach is directly applicable to salty samples with low volume limitations.[222,223]

The automated platform operates with 96- or 384-well plates and surface sampling has also been demonstrated (**Movie S-04**). Different wells are analyzed with a washing step in between leading to acquisition speeds of *ca.* 1 minute per sample which allows elimination of expendables. Using this robotic platform MS™, with help from Drs. Tamara Hendrickson (WSU) and Vladimir Shulaev (University of North Texas), differentiated among different strains of *Escherichia coli.* A comparison using ESI and MAI on the automated Ionique platform retrofitted on the Q-Exactive Focus Orbitrap is shown in **Fig. 4.** Three-dimensional principal component analysis (3D-PCA) was used to contrast the performance of both methods. Importantly, MAI, like ESI, is able to ionize small to large molecules giving a more complete microorganism fingerprint.[10,16,130,224] A comparison of ESI (1 μL), SAI (0.2 μL) and MAI (0.1 μL) consumed bacterial extract solution is provided in **Fig. S44**. ESI and SAI detected similar ions in the low mass range, whereas MAI (3-NBN matrix) additionally 'pulled out' the higher mass ions. Clearly, for best results, a combination of ESI and MAI is desirable. Overall, over 5000 bacteria extract samples were acquired using the automatic Ionique without indication of instrument contamination.

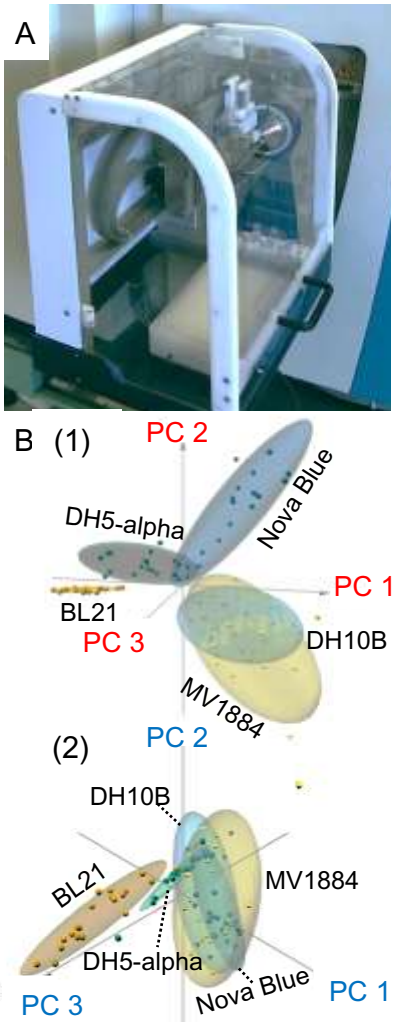


***Fig. 4**: Bacterial strain differentiation using two ionization methods MAI and ESI on the same platform and mass spectrometer (**A**) Photograph of the automated Ionique multi-ionization platform mounted on a Thermo Q-Exactive Focus Orbitrap without any further modifications (source override included in platform). (**B**) Differentiation of bacteria using a 3D-Principal Component Analysis (3D-PCA), (**1**) under standard MAI conditions 4 out of 5 E. coli strains were correctly discriminated using 3D-PCA analyses, as a comparison (**2**) typical ESI conditions, where only 2 of 5 were clearly separated.*

The Ionique platforms are able to work with Waters instruments using a MS^{TM} inlet tube that is readily retrofitted onto the skimmer cone of the commercial Z-spray source (**Fig. S45** and **Movie S-04**). One of the advantages of using these instruments for automation experiments is the availability to perform IMS which adds a second dimension of separation in addition to *m*/*z*. As one example, the well-defined mixture of β-amyloid (1-42) and (42-1) prepared in the ratio of 1:1 (*v*:*v*) was analyzed using MAI-IMS-MS (**Fig. 5**). Both β-amyloid (1-42) and reverse peptide (42-1) showed the expected[23] multiply charged ions and are indistinguishable from each other as shown in the obtained mass spectrum (**Fig. 5A**). Of course, MS alone cannot distinguish one isomer from the other. However, two different IMS drift time distributions were observed for each of the respective multiply charged ions indicating the difference in the collisional cross section of the isomers, present in the mixture, leading to effective gas-phase separation. This is accomplished using the Ionique automated platform in *ca.* 1 minute plus software interpretation time. While this is sufficient speed for an academic lab, it is not for numerous industrial applications of importance. For this reason, we focused efforts on enhancing the speed of analyses based on both SAI (**Fig. S46**) and MAI (**Fig. 4** and **S44**) with and without the use of a laser.

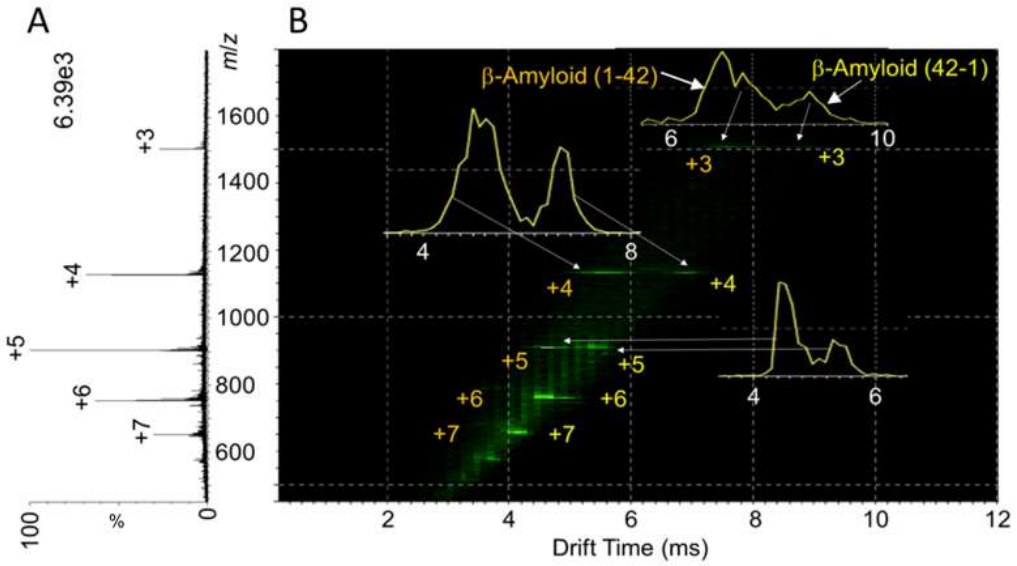


***Fig. 5**: Ionique automated MAI-IMS-MS of an isomeric mixture of β-amyloid (1-42) and reverse peptide (42-1) on an IMS-MS instrument: (**A**) total mass spectrum; (**B**) 2-dimensional plot of IMS-MS with the color code indicating the ion abundances of the respective charge states detected from +3 to +7. Insets: extracted drift time distributions separating the isomeric composition by as little as the cross section. Data acquired on Waters SYNAPT G2S mass spectrometer using the MS™ automated multi-mode Ionique platform[184] mounted on the ESI source using the retrofitted MS™ inlet tube modification and the matrix 3-NBN (100 mg of 3-NBN was prepared in 3 mL of 3:1 (v:v) ACN:water). The drift time results compare well with those obtained by LSI-IMS-MS on a SYNAPT G2.[40]*

In one approach, we tested the use of a laser to ablate solutions in well plates. This approach uses an X,Y,Z stage in combination with an in-house 3D printed well plate holder which moves the samples through the path of the laser beam. A similar setup is shown in Movie **S-05**. An Nd:YAG laser at various wavelengths was used to generate a mist from each well plate that, at least in part, was captured in the extended inlet tube for ionization by SAI. The laser can be arranged in TG (**Fig. 6** and **Fig. S47**) and RG (**Fig. S48**). Using TG, special well plates with sufficient transmission at the laser wavelength were required. The TIC of three different repetition rates using a laser at 532 nm and firing the laser at 1 Hz is provided in **Fig. 6B**. Only low analyte ion abundance was observed at the lowest settings but was augmented with increased laser repetition rates. Seven samples are analyzed in 30 seconds with reasonable ion abundances. There was a constant challenge with sample-to-sample carryover which is not unexpected for direct ionization approaches including ESI and AP-MALDI and their newer developments typically summed into 'ambient ionization'.[96,225,226] We hypothesized that *vacuum* ionization methods should not suffer these same issues. For this reason, we increasingly turned our attention to *vacuum* ionization, which continued to proceed in parallel to the *inlet* ionization developments but were technically harder to manage and solve.

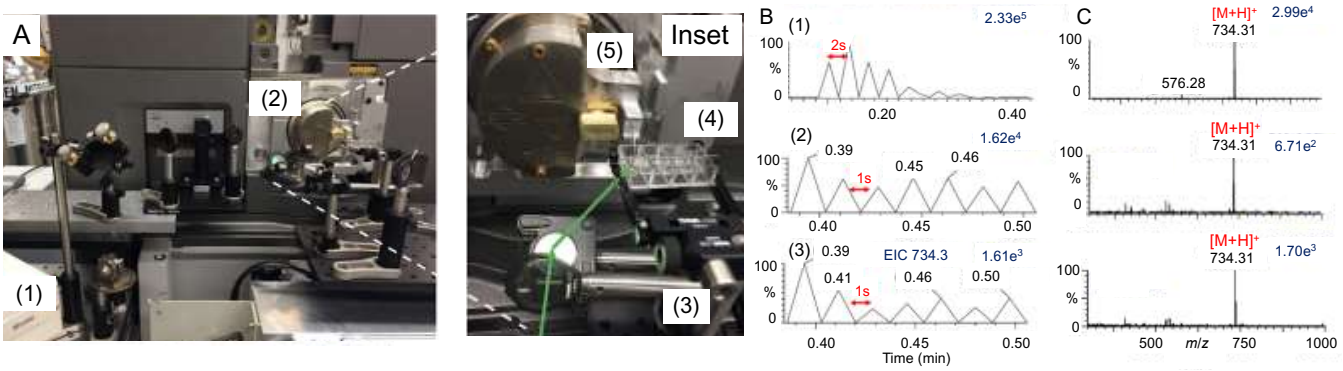


Fig. 6. *Ultra-fast analysis using TG laser ablation-SAI-MS on an IMS-MS instrument: (**A**) Photographs of the source setup with inset: green arrow provides the direction of the laser through the bottom of the optical well plates. (**1**) Nd:YAG laser ablation of the solvent (methanol) containing the analyte (erythromycin) in the wells, (**2**) well plate in close proximity of a customized inlet entrance, (**3**) automated X,Y,Z stage equipped with the custom built plate holder to secure the well plate, (**4**) sample solutions in well plate, (**5**) inlet entrance modified MS™ inlet tube; (**B**) TIC's and (**C**) mass spectra at different speed of acquisitions. The Nd:YAG laser was operated at 532 nm wavelength with a repetition rate of 1 Hz. Scan times of <1 second provide good ion abundance with increased laser repetition rates. Multiply charged ions are shown in **Fig. S45**. Data acquired with a mounted on a commercial Z-Spray source retrofitted with a heated inlet tube on a Waters SYNAPT G2S, for specific details and additional results refer to **Fig. S45-S48**.*

- *Vacuum ionization sources – probe based*

Because of carryover issues limiting speed of analysis with AP sample introduction approaches, we spent additional effort on sample introduction directly into *vacuum.* The matrix 3-NBN is exceptionally sensitive for drugs, peptides, and protein analyses.[5–11,133,227] However, *v*MAI using the commercial intermediate pressure sample introduction into the vacuum of the mass spectrometer is not very useful because sample loading requires nearly two minutes, and only a single sample could be loaded initially.[2,3] To circumvent the slow loading, and to provide a means to better study this ionization process and eliminate the long sample introduction, a vacuum-probe device was designed for Waters (**Fig. 7**) mass spectrometers replacing the commercial API sources. Single sample introduction via a probe into the vacuum of the mass spectrometer takes less than 10 seconds, even by inexperienced operators, without need of any additional pumping.[12,13,161] Abundant analyte ions with little background were achieved from low femtomoles of sample loaded without use of a laser, high voltage, or applied heat. Importantly, biological fluids, analyte in buffers, and high concentration samples were acquired without any carryover or any indication of instrument contamination.[8–11,161]

These studies suggested that the *vacuum* ionization approach virtually eliminates instrument contamination even with dirty samples because almost all of the nonvolatile materials remain on the probe surface to be removed from the instrument. Likewise, carryover is eliminated even with highly concentrated samples, the detection limit without optimization is a few attomoles, and im-

portantly, the method is suitable for surface analysis of small (<1 mm^2) sample areas as was previously shown on commercial intermediate pressure sources.[2–4,133] Because there is no physical contact and no additional energy is added to ablate the material from a surface, contrary to, *e.g.*, MALDI, only materials volatile enough sublime or evaporate leave the probe, except for the minute amount emitted as charged matrix:analyte particles. Another key advantage of fast sample introduction is

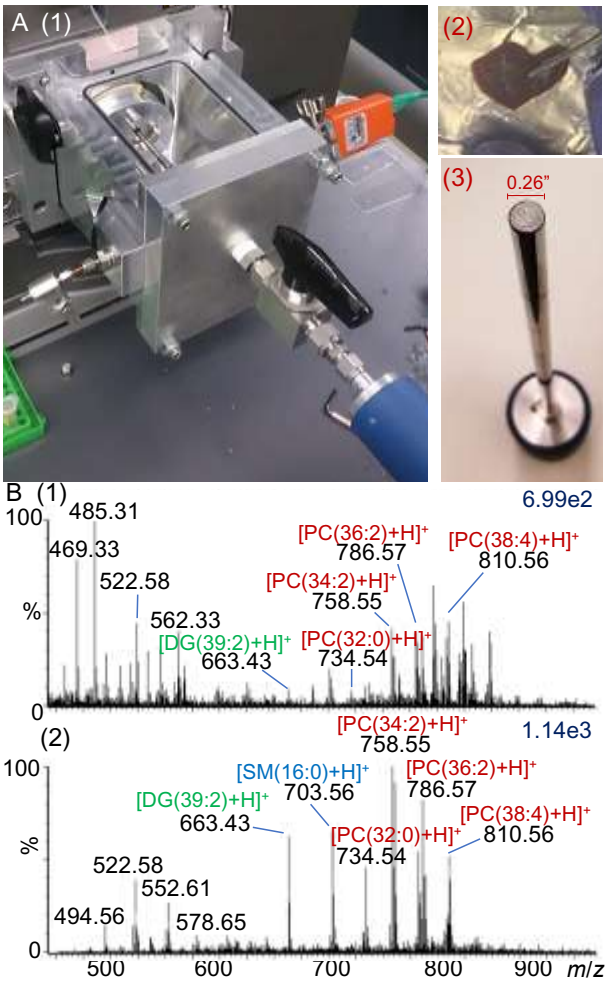


***Fig. 7**: Use of a vacuum-probe source design for vMAI-MS. (**A**) Photographs: (**1**) the source housing open and the approximate position of the probe relative to the entrance to mass spectrometer, (**2**) swine liver tissue to be analyzed, (**3**) length and diameter of the probe rod and the display of the matrix:analyte sample such as extracts dried on the probe tip; (**B**) MAI-MS: (**1**) an extraction of the tissue, the extracted was mixed with 3-NBN in acetonitrile (3:1 v/v) 0.1 µL was used for the acquisition and (**2**) tissue analyzed directly after being exposed with the matrix solution and briefly dried. Data acquired on a SYNAPT G2 mass spectrometer. Tentative m/z assignments are based on published work.[228–231] DG: diacylglycerol; PC: phosphatidylcholine; SM: sphingomyelin.*

that volatile matrices are applicable such as coumarin and methyl 2-methyl-3-nitrobenzoate which sublime before reaching the ionization region using the commercial mechanical MALDI sample introduction to the vacuum of the same mass spectrometer.[3] This is expected to enhance the sensitivity by (**1**) not 'losing' the sample due to improper source introduction and (**2**) improving desolvation of the matrix from the charged clusters. Because the ionization is now also robust one can analyze without concerns materials directly from biological matrices, buffers, etc. Examples include tissue,[9,10] polymers,[170,232] proteins,[11,43] bacteria extracts, drugs and their starting materials (**Fig. S49**)[132] to name a few. Even proteins are detected, and charge states can be extracted to give an additional value of confirmation using the drift time values associated with their respective cross sections previously determined by an ESI approach.[233–236]

For more meaningful research related to vMAI in the MS™ lab and seeing the promise of the vacuum probe method on the Waters SYNAPT G2, MS™ subsequently built a vacuum-probe device for a Thermo Q-Exactive Focus Orbitrap mass spectrometer and demonstrated low attomole detection limits and no carryover when loading

10^{11} times more analyte, so long as the probe tip was replaced. These results were acquired with 3-NBN as matrix using ion extraction voltage to direct ions into the 'S' lens of the Q-Exactive Focus. The drug fexofenadine was detected with S/N >10/1 from only 7 attomoles, and from 100 attomoles applied directly to a metal surface as a demonstration of analysis of a microscopic deposit.[10,11,13]

Similar to MALDI, with certain compounds such as peptides and drugs, cleaning the surface of the sample plate after analysis so that the analyte is not observed in subsequent analyses can be difficult. For example, with erythromycin placed on a metal probe at 1 μM concentration and dried, multiple washing steps with several common solvents failed to remove the sample sufficiently so that it is not observed in significant abundance in a subsequent vMAI analysis where only matrix solution is added. However, using the solvent used with vMAI, but without the matrix added, and applying it to the surface followed by nanoESI did not detect the drug.[13] These and other results reiterate that the matrix plays a key role in dissolution (solvation) of the analyte from a surface. Adding the matrix on top of the tissue (as would be the case of *e.g.*, a biopsy sample) results in a lower abundance of ions but a wealth of signals relative to a typical Folch extract,[237,238] as is shown in **Figure 7B**. The use of the *v*MAI probe demonstrated its potential for rapid analyses, *e.g.*, 2.4 seconds per sample.[13] This is possible because there is no physical contact and ionization ceases when not in close proximity to the ion transmission region. In this experiment, nothing can be learned about true speed and potential carry-over issues from one sample to the next because the same sample was acquired multiple times by moving the probe in and out of the ion transmission window.

Ionization is rather limited for some materials using MS such as less basic compounds, including carbohydrates and synthetic polymers requiring the addition of salts to obtain good results, and in some cases the use of binary matrices to enhance detection. Using the vacuum-probe on the SYNAPT G2S, we analyzed and confirmed in a "walk-up" manner synthesized carbohydrates from our collaborator Dr. Nguyen's group (WSU) (**Fig. S50**). Yet another specific example is shown in **Fig. S51** in collaboration with Dr. Scott Grayson's lab (Tulane University) in which the graduate student, McKenna J. Redding, travelled to WSU. A combination of tetrabenzylidene G1 and octabenzylidene G2 bis-MPA dendrimers[239,240] were readily analyzed with the addition of barium solution and 3-NBN using the vacuum-probe on the SYNAPT G2S. In collaboration with Dr. Barbara Larsen, branched polymers were readily differentiated using the vacuum-probe vMAI-IMS-MS approach without the time consuming clean up between samples.[170] While highly charged polymer ions, important for distinguishing differences in polymer architectures using MS in combination IMS fingerprint analysis have been difficult with

the matrix 3-NBN, other MAI matrices have been successful.[214,241] Another improvement was the introduction of use of divalent cations.[232,241] Binary matrices and salts may open up many more research opportunities in the future. Therefore, with *vacuum* ionization using a simple probe device we gained the freedom to improve our understanding of materials of all sorts because of the high ion abundance *and* without worry about source and instrument contamination. Using a vacuum-probe we are finally comfortable to measure any sample because we do now know that we can't harm the instrument or cause extra work because of a dirty inlet.

A miniature vacuum-probe was recently designed, constructed, and applied with a portable mass spectrometer by Dr. I-Chung Lu group (National Chung Hsing University).[242] Peptides, drugs, and crude coffee samples have been analyzed on this mass range limited mass spectrometer with surprisingly little chemical background.[221] A minimalistic vacuum/inlet-source was previously designed, enabling the analysis of samples using a simple on/off valve controlling airflow from the AP to the vacuum. This device showed portability is possible including fast analyses with simplicity and high sensitivity, and as a bonus offered insights into flexible valve designs.[92] Drug tablets have been analyzed *e.g.,* propranolol was detected, simply by adding the matrix 3-NBN and moving the tablet close enough to a mini source[84,132] on a Waters SYNAPT G2S (**Fig. S52**). The accumulated *vacuum* ionization results lead to the bold and successful research described in Section 1 of this *Perspective*.

Lesson 7:

- Information extractable from dedicated platforms is more fundamentally and analytically useful
- Unprecedented simplicity, speed, and robustness is achievable with dedicated *inlet* and *vacuum* ionization sources
- Clinical applications are within reach

Conclusion and Outlook

As a personal note, the success I've (ST) had that lead to the Biemann Medal belongs, in a great measure, to a long list of students and collaborators, a number of whom are co-authors on this *Perspective*. It has been my privilege to work with talented undergraduate and graduate students of different genders and ethnicities from around the globe. There are too many talented students to list them all here, but I'm especially indebted to Prof. Ellen Inutan, my first graduate student, and a gifted researcher who first observed vMAI. I like the saying attributed to Henry Ford; "Whether you think you can, or you think you can't--you're right",[243] because my group accomplished the most when we all believed. I am amazed at how far we have come since the first observation of multiply charged ions from what appeared to be a MALDI method, but also somewhat dismayed that not more researchers have applied and developed these technologies. I do believe that this will

change with the recent vacuum source developments by MS™. Happily, the new ionization developments have been included in a popular undergraduate textbook.[244]

Because the new ionization methods are based on heretofore unknown ionization processes, and having received almost no development, relative to well-optimized ionization methods of ESI and MALDI from over 30 years ago,[70–72,80–83,177–181,195–197,200,202,203,205–208,233–236,240,245,246] it is our hypothesis that the new methods lie at the very beginning of the technology life cycle curve. Thus, improvements are far more likely than with mature ionization technologies. Therefore, sensitivity increases, which can be expected from dedicated studies, will allow simultaneous ionization of multiple components in complex surface chemistries. Our expectation is that in the immediate future we can improve the detection limit of vMAI by physical source constructions best suited for the new ionization processes making it a low-cost and rapid method for robust, laser-free, low-resolution, imaging with high sensitivity. Further, understanding the role the matrix plays in nearly all ionization methods such as ESI, MALDI, DESI, as well as the novel ionization processes described here, in displacing compounds from a surface should further improve molecular (surface) analysis. We hypothesize that monolayer molecular and bulk surface analysis can be made into a routine technology using readily available mass spectrometers through application of the exceptionally unique vMAI technology combined with the latest vacuum source technology. Not only can surfaces be probed on a molecular level, but potentially imaged with approximately 100 μm^2 spatial resolution without use of a laser. Defects caused by chemical contaminants promptly can be analyzed in seconds without a laser, high voltages, desolvation gases or any of the traditional means, an important point for ionizing valuable samples. More broadly, the new processes permit simple and inexpensive analyses, by a novice, and through dedicated developments of robust instruments producing quality data in a wide range of applications. Relevant to Dr. Klaus Biemann's legacy,[247–252] we will put our best efforts forward in making MS analysis of even large proteins, as well as other demanding molecules, fast and easy to achieve, even by a novice.

Overall Lesson:

- Optimization of ESI and MALDI, led to broad applicability that was unimaginable at the beginning
- Similar advances in science is not unexpected for the new ionization processes

ASSOCIATED CONTENT

Supporting Information

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

The Supplemental PDF file contains additional details to the experimental information, movies, and Figures supporting the description of this text.

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ACKNOWLEDGMENT

Trimpin has been gifted by a wonderful body of all sorts of teachers from parents, family, school teachers, professors, colleagues, students, and postdocs. The authors are grateful for the generous support from NSF CAREER 0955975, NSF CHE-1411376 (to ST), NSF Phase II 1556043 including TECP and NSF SBIR Phase I 1913787 (to CNM and ST), CHE-1112289 (to CNM), CHE-1500201 and CHE-1904584 (CNV), CHE-1904670 (MKP), CHE-1807358 (SMG), as well as Eli Lilly Young Investigator Award in Analytical Chemistry, DuPont Young Professor Award, and BASF financial support (to ST), WSU Schaap Faculty Scholar, OVPR Grants Plus (to ST) and CVRI ISIS (to THS and ST), WSU Rumble Graduate (to EDI and BW) and undergraduate fellowships (to ALR, CBL, and FSY). NIH R01CA095142 and NIH R01GM090270 (to ZG), NIH R01 DA011322 and DA021696 (to KM), NIH R01 GM098285 (HMN), NIH R01 HL131740 (to Y.-H. A). We also thank Dr. David Crich (UGA) for providing standards. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

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