Current Trends in MASS Spectrometry

SUPPLEMENT TO
May 2014
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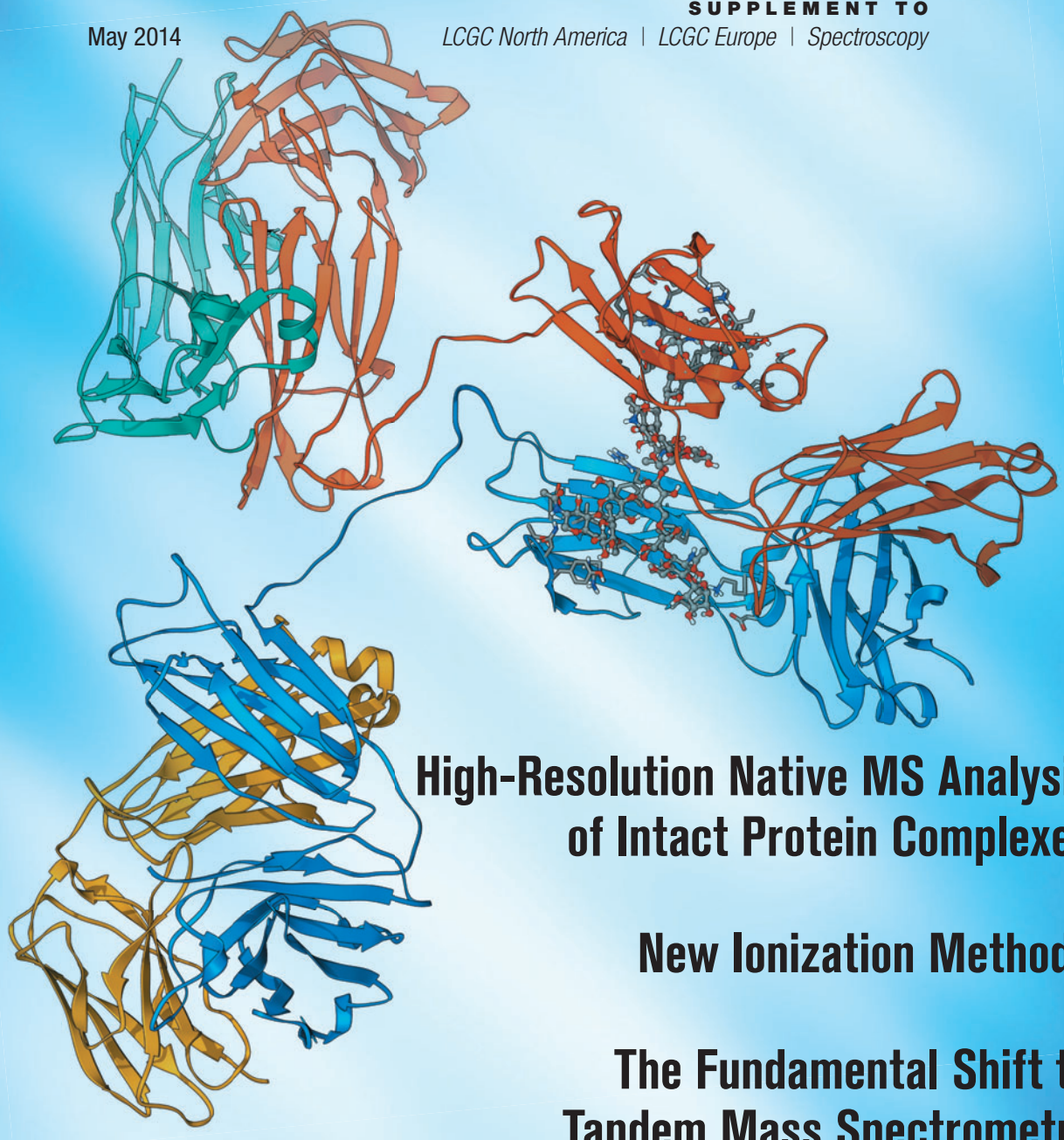

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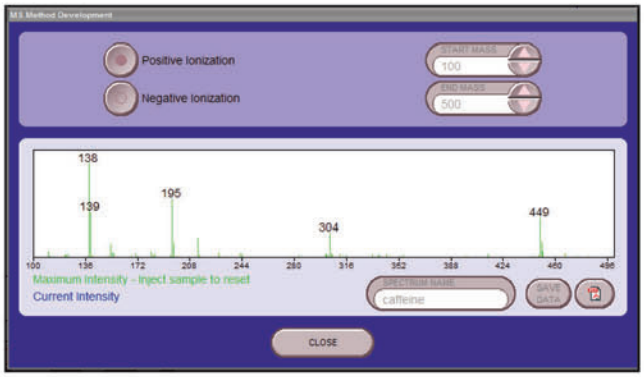

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A Convenient Alternative to MALDI and ESI

Ionization of small, large, volatile, and nonvolatile compounds with charge states nearly identical to electrospray ionization are produced from a solid matrix or solution with high sensitivity utilizing the vacuum inherent with any mass spectrometer. With the proper matrix, analytes can be analyzed from ambient conditions or by direct introduction into vacuum. The ion source is simply the inlet to the mass analyzer. The new ionization methods have been interfaced with liquid chromatography, used for imaging tissue at atmospheric pressure or in vacuum, demonstrated for high-throughput analyses, applied for fast defect analysis, and shown to be compatible with electron transfer dissociation and ion mobility spectrometry–mass spectrometry. With the proper matrix, even large proteins are converted to gas-phase multiply charged ions without application of an external source of energy other than subatmospheric pressure. This latter method has great potential for extending mass spectrometry to areas such as clinical analysis where cost, robustness, and simplicity are important assets.

Sarah Trimpin, Beixi Wang, Corinne A. Lutomski, Tarick J. El-Baba, and Bryan M. Harless

In matrix-assisted laser desorption–ionization (MALDI), predominantly singly charged gas-phase molecular ions are produced from analytes incorporated into a matrix. However, laser ablation of a common MALDI matrix at atmospheric pressure produced ions with higher numbers of charges common with electrospray ionization (ESI). Fundamental studies directed at understanding how multiply charged ions are produced by a process that is nearly identical to MALDI, with the main difference being that ion extraction voltage is removed or lowered, led to a series of new ionization methods given the general terms *inlet* and *vacuum ionization*. The laser was found to not be necessary for *matrix-assisted ionization* (MAI). Ionization occurs when the analyte incorporated in a small molecule matrix is introduced into a heated inlet tube linking atmospheric pressure and the vacuum of the mass spectrometer. MAI has now been extended to operate in vacuum using laser ablation similar to MALDI, but producing ions with ESI-like charge states. Here, the laser is also not a requirement. Matrices have been discovered that efficiently lift solid-phase molecules into the gas phase as ions without any external energy source when exposed to subatmospheric pressure. The development of these novel ionization processes for use in mass spectrometry (MS) is discussed. These methods are simple to use, safe, robust, and sensitive.

A Brief Evolution of Ionization Methods for Use in Mass Spectrometry

The inventions of ESI (1) and MALDI (2,3) in the 1980s led to important advances in science because they provided, for the first time, a means of characterizing minute quantities of nonvolatile compounds even in complex mixtures using MS. Before the advent of ESI and MALDI, methods such as field desorption, plasma desorption, fast atom bombardment, laser desorption, and thermospray ionization made inroads into converting nonvolatile compounds into gas-phase ions (4–19). ESI and MALDI quickly replaced other ionization methods used with nonvolatile compounds, each having strengths that complement the other. ESI produces multiply charged ions from compounds in solution having multiple basic sites, is compatible with liquid separation methods, and is capable of analyzing large molecules on mass-range limited mass spectrometers. MALDI replaces the solvent in ESI with a solid small-molecule matrix and produces predominatly singly charged ions upon laser ablation of the matrix. MALDI operates from surfaces and has excellent sensitivity, and singly charged ions simplify analysis of complex materials such as polymers. However, MALDI is incompatible with liquid chromatography (LC) except under certain conditions (20). Because of deficiencies in ionization of low-polarity compounds using ESI, atmospheric-pressure chemical ionization (APCI) has gained in popularity as an alternative LC–MS ionization method (21,22).

In the past decade, numerous innovative new sampling methods have been developed under the umbrella of "ambient ionization" (23–31). The mechanisms by which ions are formed in these methods are either gas-phase ion–molecule reactions as in APCI (32) or charged droplets as in ESI (33,34). Desorption electrospray ionization (DESI) (35,36) appears to have elements of both. The ambient ionization methods minimize sample preparation (31) and are associated with direct ionization in which chromatography is not used. For complex samples, high resolving power MS or ion mobility spectrometry (IMS) enhances (37) or replaces (38–40) the chromatographic separation, but ion suppression issues are more pronounced with the direct methods. Progress has been made in coupling MS with IMS in which gas-phase ions are separated in time before MS analysis (41–43). Commercial IMS-MS instruments are now available providing rapid and high sensitivity analysis. The most prominent IMS-

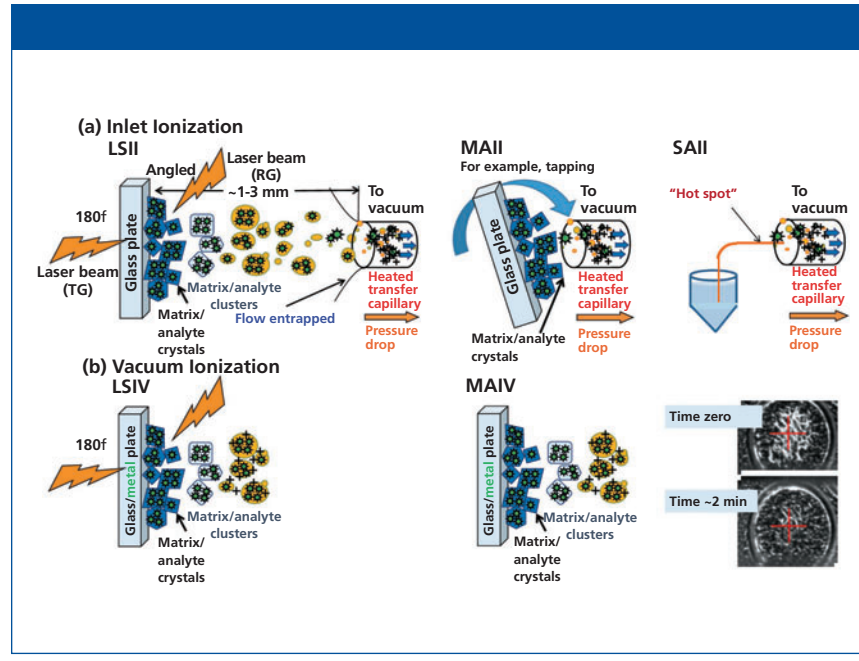

Figure 1: Schematic representation of (a) inlet ionization: laserspray ionization inlet (LSII), matrix-assisted ionization inlet (MAII), and solvent-assisted ionization inlet (SAII); and (b) vacuum ionization: laserspray ionization vacuum (LSIV), matrix-assisted ionization vacuum (MAIV). Lower right: screen shots from the vacuum MALDI source capturing the matrix–analyte sample as soon as indexed to the final position (top) and after the MAIV experiment was completed by simply letting the sample sublime (bottom).

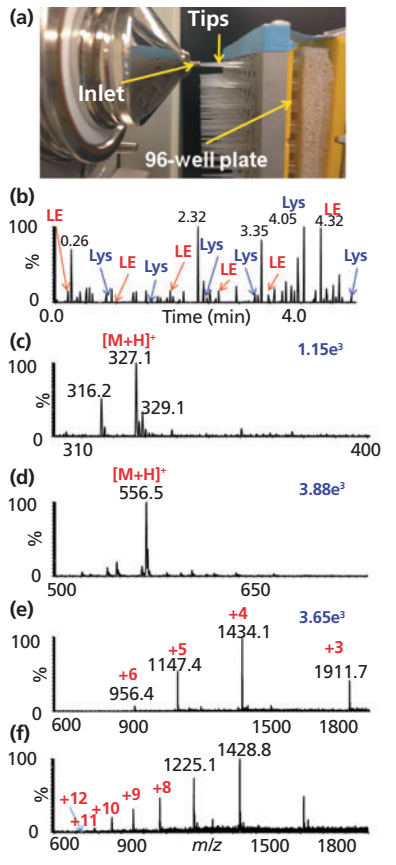

Figure 2: SAII-MS: (a) photograph of 96-well pipette tip holder mounted on an automated *xyz*-stage and aligned with the commercial inlet tube to move the solution containing the sample right in front of the inlet in which the ionization event is initiated by the vacuum draw of the mass spectrometer. The source housing is removed and operation overridden for convenience entrance. (b) Total ion chronogram of 42 samples with 42 blanks in between each sample acquired within 5 min, (c) mass spectrum of clozapine (MW 326), (d) mass spectrum of leucine enkaphalin (MW 555), (e) mass spectrum of bovine insulin (MW 5730), and (f) mass spectrum of ubiquitin (MW 8560). Data were acquired using the Thermo Fisher Scientific LTQ Velos mass spectrometer. Adapted from reference 64 with permission. Copyright 2014 American Chemical Society.

MS instrument uses traveling wave ion mobility spectrometry (TWIMS) technology (44). Improvements in TWIMS have enabled the separation of isomeric species (39,45,46), which is unavailable in MS alone.

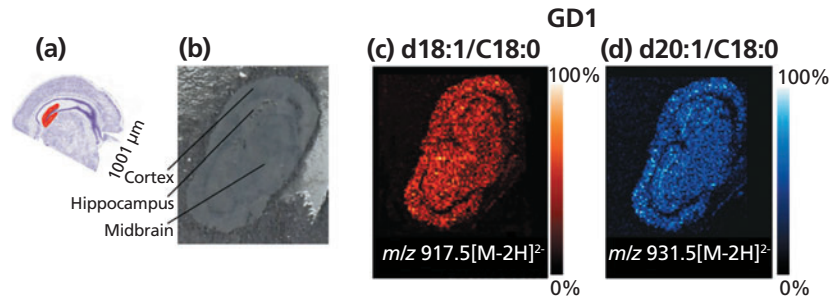

Figure 3: Mass spectrometry imaging using LSII with the laser (N_2) aligned in transmission geometry: (a) photograph of a mouse brain tissue section from the Allen Brain Atlas (84), (b) experimental mouse brain tissue section of 10 µm thickness precoated with matrix 2,5-dihydroxybenzoic acid and spray coated with matrix 2,5-dihydroxyacetophenone, and (c,d) LSII images of the doubly deprotonated ions of disialoganglioside GD1 at *m/z* 917.5 and 931.5. Data were acquired on a Thermo Fisher Scientific LTQ Velos mass spectrometer at an inlet temperature of 450 °C. Adapted from reference 66 with permission.

Within ambient ionization there are ESI-based approaches capable of ionizing nonvolatile compounds such as DESI, laser ablation ESI (LAESI), and matrix-assisted laser desorption ESI (MALDESI), as well as those that ionize vaporizable compounds using APCI such as atmospheric solids analysis probe (ASAP) (25) and direct analysis in real time (DART) (24). A number of ambient methods are suitable for MS imaging (47). These approaches create a molecular image from the surface of interest (48). Ambient ionization methods also show promise in the characterization of proteins, but because these approaches operate on mass spectrometers with limited mass range, they must produce multiply charged ions in sufficient abundance for imaging (49). These atmospheric-pressure imaging approaches currently suffer either from limited ionization sensitivity, low spatial resolution, or both.

New ionization methods may be required to achieve a step-change improvement in surface characterization (50). One such new ionization method was discovered in an attempt to develop field-free transmission geometry atmospheric-pressure MALDI for tissue imaging (51,52). This new ionization method was termed *laserspray ionization inlet* (LSII) (Figure 1a, left) (53). Instead of observing the expected singly charged ions of peptides applied to a glass slide in a solution of the MALDI matrix, 2,5-dihydroxybenzoic acid (2,5-DHB), multiply charged ESI-like ions were observed from the dried solid-state matrix. The initial expectation that ionization was the product of laser ablation gave way to fundamental studies that demonstrated ionization occurred when the matrix–analyte particles or molten droplets ablated from the surface entered the heated inlet tube linking atmospheric pressure and the first vacuum region of the mass spectrometer (54–57). Because identical mass spectra were obtained by physically introducing the matrix–analyte sample into the heated inlet tube rather than by laser ablation, the more general term *matrix-assisted ionization inlet* (MAII) was used (Figure 1a, center) (58). Interestingly, Leonard Nyadong working in Alan Marshall's group with LSII on a high resolution Fourier transform MS instrument independently discovered that the laser was not necessary for ionization (59).

Pagnotti and McEwen (60) discovered that a solid matrix is not a requirement for ionization to occur in a heated mass spectrometer inlet tube. Solvents also produce abundant ions having charge states nearly identical to those observed in ESI, and the sensitivity of this method, termed *solvent-assisted ionization inlet* (SAII) (Figure 1a, right), is comparable to or better than ESI at simi-

lar flow rates (60). SAII works well with microliter (61) and nanoliter (62) flow LC–MS (7 ng of bovine serum albumin tryptic digest injected on column) (62), and low femtograms for steroids (63). An example of multiplexed SAII, capable of analyzing 42 samples in about 5 min, is displayed in Figure 2 (64). SAII is a liquid introduction variant of MAII operating without a laser or a voltage.

Thus, LSI was the first of a family of ionization methods in which the inlet tube becomes the ion source (Figure 1a). These methods encompass samples in solid matrix common to MALDI and in solution common to ESI. Because LSI uses laser ablation and produces highly charged ions similar to ESI, but directly from the solid state without applied voltage, it is especially useful for analysis of small areas. LSI combines the attributes of MALDI, including speed of analysis and high spatial resolution for imaging, and those of ESI, including operation from atmospheric pressure, extending the mass range of high performance mass spectrometers, improved structural information through enhanced fragmentation (such as electron transfer dissociation [ETD]), and improved IMS separation. Thus, LSI extends the mass range of compounds that can be analyzed using atmospheric-pressure laser ablation with new high performance, but mass-to-charge (m/z) range limited mass spectrometers. For example, using LSI, ubiquitin (MW 8561) was mass measured following laser ablation directly from the solid state using a mass spectrometer with 100,000 mass resolution, <5 ppm mass accuracy, and an upper mass limit of 4000. Sequence information was obtained from this protein using ETD fragmentation of the +11 charge state (53). LSI-ETD was also used to identify the endogenous *N*-acetylated myelin basic protein fragment peptide directly from mouse brain tissue (65). Multiply charged ions help IMS-MS differentiation of isomers and LSI provides a means of producing multiply charged ions directly from surfaces (45). The spatial resolution from laser ablation in transmission geometry (backside ablation), as well as the softness of LSI, enabled the imaging of fragile gangliosides directly from mouse brain tissue (Figure 3) (66). Transmission geometry laser ablation has also been successfully coupled to other ionization methods and used to image and characterize handwriting and dusted latent fingerprints (67). Thus, the inlet ionization methods operating from atmospheric pressure provide a viable alternative to ESI and MALDI and possess unique attributes.

However, the story of the initial finding that multiply charged ESI-like ions can be generated from a solid matrix does not end with inlet ionization, which occurs within a heated inlet tube linking a higher and a lower pressure region. Fundamental studies intended to understand the mechanism of inlet ionization (68) led to extending the LSI approach to producing highly charged analyte ions in vacuum ion sources, normally used for MALDI, without the need for a heated inlet. Because a heated inlet tube was not required, these methods were termed vacuum ionization (Figure 1b) to differentiate them from inlet ionization (Figure 1a). Matrices and instrumental conditions were found

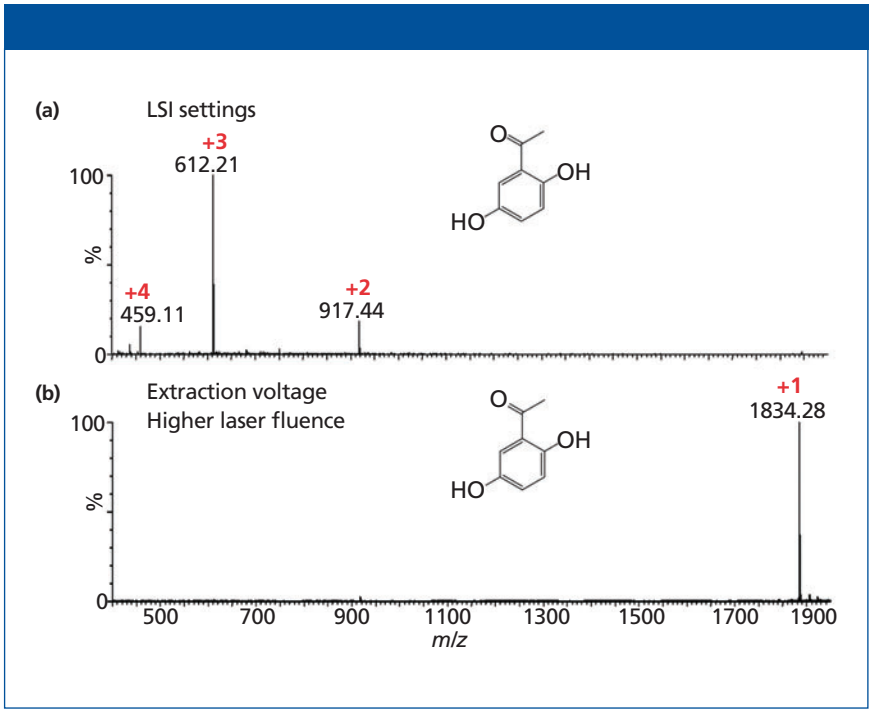

Figure 4: LSIV mass spectra of *N*-acetylated myelin basic protein fragment (MW 1833) using the same matrix: (a) soft settings, similar to commercial ESI settings (no plate extraction voltage and lower laser power); (b) harsh settings, similar to commercial MALDI settings (plate extraction voltage 20 V and high laser power). Data were acquired using the intermediate-pressure vacuum MALDI source of a Waters IMS-MS Synapt G2 mass spectrometer. Structure of matrix compound 2,5-dihydroxyacetophenone provided as insets. Adapted with permission from Springer Science and Business Media from reference 70.

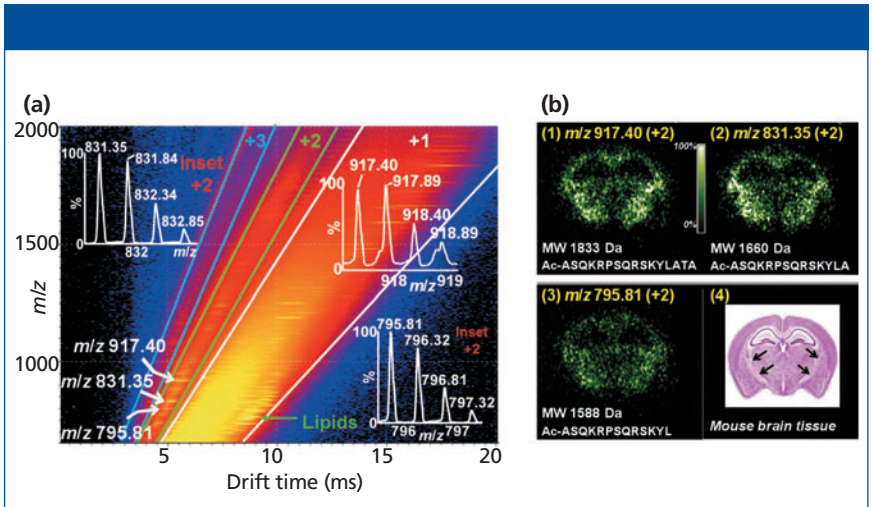

Figure 5: MS imaging using LSIV with the laser (Nd:YAG) aligned in reflection geometry: (a) LSIV-IMS-MS of delipified mouse brain tissue: two-dimensional plot of drift time versus *m/z* insets with isotopic distributions of doubly charged *N*-acetyl myelin basic protein fragment peptide ions at *m/z* 917.40, *m/z* 831.35, and *m/z* 795.8. (b) Images of endogenous peptides with *m/z* 917.40, 831.35, and 795.81 are related to a photograph of a mouse brain tissue section (lower right) (85) with regions of high myelin content indicated by black arrows. Data were acquired using the intermediate-pressure vacuum MALDI source of a Waters IMS-MS Synapt G2 mass spectrometer. Adapted from reference 74 with permission. Copyright 2012 American Chemical Society.

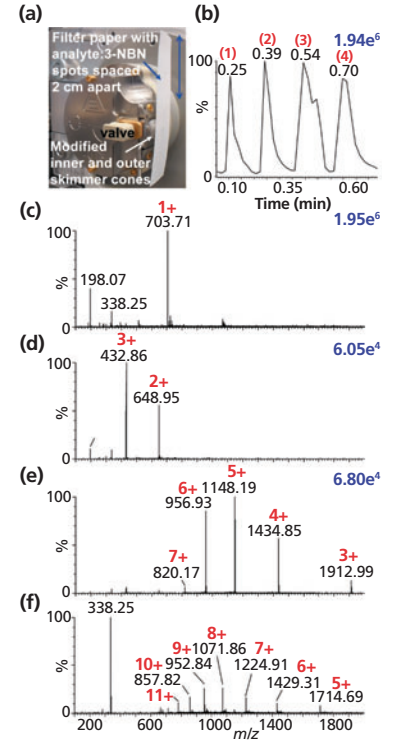

Figure 6: MAIV-MS. (a) Photograph of a strip of filter paper spotted with four different matrix–analyte samples. The filter paper adheres to the skimmer cone by the vacuum draw of the mass spectrometer. (b) Total ion chromatogram of the sequential acquisitions; mass spectra of (c) sphingomyelin (MW 702), (d) angiotensin I (MW 1295), (e) bovine insulin (MW 5730), and (f) ubiquitin (MW 8560). Data were acquired using a widened inner cone on the Z-spray ion source of a Waters IMS-MS Synapt G2 mass spectrometer. Adapted from reference 77 with permission. Copyright 2012 American Chemical Society.

that produce charge states identical to LSII and MAII using the commercial intermediate pressure MALDI source of a Waters Synapt G2 mass spectrometer (69). Interestingly, using the same matrix–analyte sample preparation with the common MALDI matrix 2,5-dihydroxyacetophenone, either MALDI-like singly charged ions or LSI-like multiply charged ions were obtained from *N*-acetylated myelin basic protein fragment peptide (Figure 4) by simply changing instrumental conditions and using lower laser fluence (70). Similar results were reported for LSII using a commercial atmospheric-pressure

MALDI source (71).

This discovery led to a problem in nomenclature because the exact same laser ablation process is used to produce either singly or multiply charged ions from a matrix commonly used in MALDI. To distinguish these processes, laser ablation under vacuum that does not require a heated inlet was termed *laserspray ionization vacuum* (LSIV) (Figure 1b, left) (69). It seems clear that by changing only the instrumental conditions, a different regime of ions is selected, producing ions of differing charge states. If the multiply charged ions are formed from the same process that produces these ions in LSII, then the laser may similarly not be directly involved in ionization under vacuum conditions. In this case, observation of multiply charged ions is not a MALDI process, or MALDI of multiply charged ions does not involve photochemical ionization. We suggested that for nonvolatile compounds, the ionization mechanism of MALDI, LSIV, LSII, MAII, ESI, and other methods applied to ionize nonvolatile compounds are mechanistically related (70).

The new ionization discoveries were achieved because of the simple concept that producing charged matrix particles or droplets is common in nature and that multiply charged ions will be observed from the charged particles or droplets if the matrix evaporates or sublimes in the time frame available before ion separation and detection (70). In other words, just as in ESI, charged particles must be generated and desolvation of the charged particles must occur. In a vacuum MALDI experiment, the laser provides the energy necessary for generating the charged particles and matrix evaporation. However, the time available before mass analysis begins in a MALDI-time-of-flight (TOF) mass spectrometer is extremely short, limiting the ability of all but small clusters to desolvate. It has been suggested that the energy available and time limitation in MALDI-TOF limit the number of charges observed on bare ions because the small clusters that are able to desolvate have few charges (70). By finding a matrix compound, 2-nitrophloroglucinol (2-NPG), that has sufficient absorbance at the laser wavelength

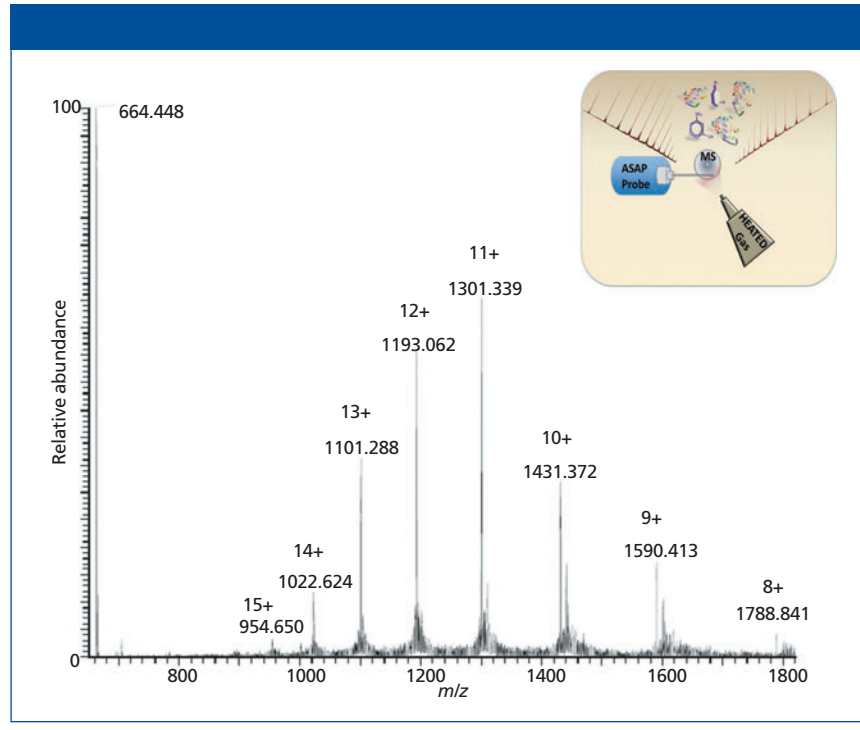

Figure 7: Mass spectrum of lysozyme (MW 14,300) using the atmospheric solids analysis probe for matrix (3-NBN)-analyte sample exposure in front of the inlet aperture. Data were acquired on a Thermo Fisher Scientific Orbitrap Exactive mass spectrometer with an inlet temperature of 250 °C, heated auxiliary gas (N_2) of 90 °C, and 3 kV applied to the heated ESI probe. Scheme for MA-ASAP acquisition provided top right. Adapted with permission from Springer Science and Business Media from reference 78.

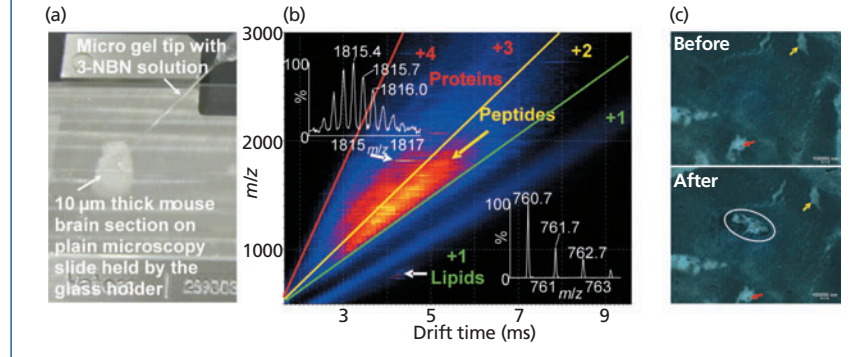

Figure 8: MAIV-IMS-MS analyses of a mouse brain tissue section: (a) photograph of procedure applying with a micropipet tip the matrix (3-NBN) solution to the surface of a mouse brain tissue section adhered to a glass microscope slide; (b) 2D plot of drift time versus *m/z* of ions depicting separation of compound classes by charge, size, and shape of lipids (inset charge state +1), peptides, and proteins (inset charge state +4); (c) photograph of the mouse brain tissue microscopy before and after exposure to the vacuum for ionization and analysis in which the circled area highlights the tissue region that was analyzed in (b). The colored arrows are provided to guide the eye. Data were acquired using soft settings from Figure 4a on a commercial vacuum MALDI source of a Waters IMS-MS Synapt G2 mass spectrometer with the laser off. Reproduced from reference 38 with permission. Copyright to the American Society for Biochemistry and Molecular Biology.

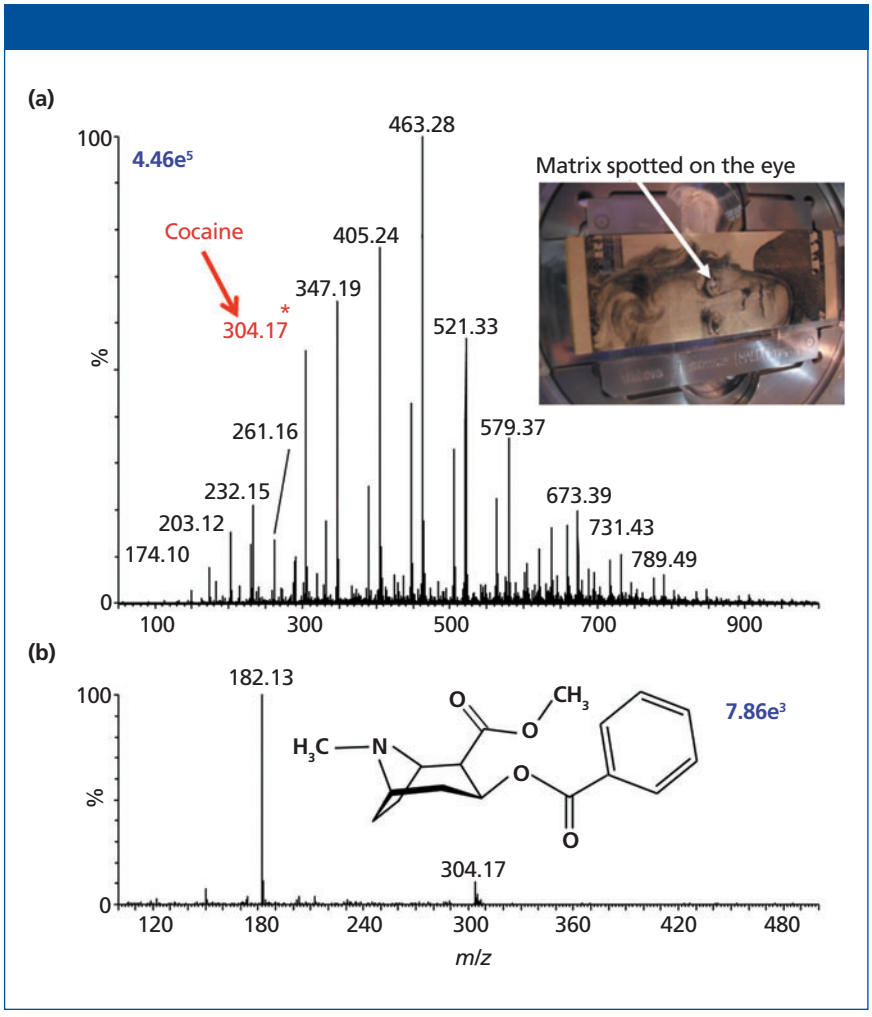

Figure 9: Cocaine (MW 303) and polypropylene glycol, both detected as singly charged ions, directly from a specific location (eye of the president) of a $20 bill by spotting matrix 3-NBN solution to the area of interest. (a) Mass spectrum obtained when the matrix was spotted on the eye of the bill and introduced to the vacuum; (b) MS-MS spectrum and structure of cocaine peak selected at *m/z* 304. Data were acquired using the intermediate-pressure vacuum MALDI source of the Waters IMS-MS Synapt G2 mass spectrometer. Adapted with permission from Springer Science and Business Media from reference 76.

and readily evaporates or sublimes, it was demonstrated that stable multiply charged ions can be generated in a MALDI-TOF experiment (72).

In intermediate-pressure MALDI instruments, the mass analyzer is remote from the ionization region so that the time available for desolvation of the charged matrix particles or droplets produced by laser ablation is orders of magnitude greater than in MALDI-TOF. Desolvation is also enhanced by collisions, rf fields, and the higher pressure used in these instruments (68–70,72–74). Thus, ESI-like charge states are produced by the 2-NPG matrix as well as a number of other matrix compounds upon laser ablation. Therefore, the experiment is similar to MALDI but a combination of a more volatile matrix and tuning conditions favorable to desolvation aid in producing highly charged ions with high sensitivity. Producing ESI-like charge states from mouse tissue sections with the spatial resolution from the laser shows great promise for imaging MS experiments using intermediate-pressure ion sources (Figure 5) (74).

Extending the concept that a laser is not necessary to produce ions in LSII leading to the discovery of MAII, we examined a large number of small molecules seeking matrices that produce ions in an inlet with low thermal requirements. With matrices that sublime in vacuum, one should only need to produce charged matrix particles to observe bare ions. During this search, 3-nitrobenzonitrile (3-NBN) was found to spontaneously produce ions from analyte, including large proteins, when prepared similar to MALDI or LSIV matrix–analyte and introduced to vacuum conditions. It is known that 3-NBN is triboluminescent when fractured (75). *Triboluminescence* is caused by a discharge occurring between two fractured surfaces carrying opposite charge. Thus, if placing the 3-NBN matrix under vacuum conditions could lead to particle ejection from expanding gases such as sublimation of the matrix or evaporation of included solvent within cavities, the fracturing process could produce highly charged gas-phase matrix–analyte particles by the same process that produces the conditions for triboluminescence.

Interestingly, 3-NBN has no acidic hydrogen atoms to donate protons to the analyte, suggesting that the observed gas-phase ions were already charged in the solid matrix. While ESI and all of the inlet and vacuum ionization methods (Figure 1b, right) produce similar charge states for gas-phase ions, MAIV must transfer ions directly from the solid state. Visualizing the process using a vacuum MALDI source (Figure 1, lower right) with a magnifying camera, one only observes that the solid matrix slowly disappears while ions are being observed.

The method in which a matrix–analyte sample is introduced to the vacuum of a mass spectrometer to produce gas-phase ions for analysis by MS is extremely simple and highly sensitive. However, using traditional MALDI plates to introduce samples to vacuum is not efficient, as only one sample can be introduced at a time. Because the atmospheric-pressure inlet provides a small opening to the vacuum of the mass spectrometer, it is possible to produce a sealed system at the inlet to initiate ion formation (38,76). In this configuration, opening the valve that isolates atmospheric pressure from the vacuum of the mass spectrometer is all that is needed to initiate ionization. Thus, sample can be handled at atmospheric pressure and rapidly changed. However, because the atmospheric-pressure inlet is designed for gas flow — without it, ion transmission is poor.

Providing gas flow with a semi-sealed

system enhances sensitivity, and it was found that gas-permeable material such as filter paper with the matrix–analyte sample held against the commercial inlet, whether a skimmer aperture or a tube inlet with no heat, or moderate applied heat, was an efficient means of producing analyte ions using 3-NBN for MS analysis (77). A very simple means of introducing sample to vacuum is to either tap matrix–analyte into the inlet aperture similar to MAII or bring it to the inlet either wet or dry using a pipette tip. When matrix–analyte is introduced into the inlet it immediately experiences vacuum conditions and produces ions. The difference from the initial MAII method is that the inlet does not need to be an inlet tube or heated, and, in fact, an inlet that is too hot is detrimental.

The elimination of the need for a heated inlet tube means this MAIV method can, in principle, be used with any atmospheric-pressure mass spectrometer regardless of the type of inlet that is used. Because the matrix sublimes in vacuum, no matrix contamination of the instrument is expected. Thus, this extremely simple and fast method of analyses may prove to be the least contaminating and useful for long-term analyses. While heat is not a requirement, in some cases it can be important. Introducing analyte in 3-NBN into a room temperature inlet, just as with introduction using a vacuum MALDI source, produces ions for an extended time period. For some analyses, this is important as it allows time for a variety of MS^n or IMS experiments; however, for high-throughput analyses, it is desirable for abundant ions to be produced for at most a few seconds (Figure 6). Modest heat applied to the inlet achieves this goal.

For 3-NBN, atmospheric-pressure inlet temperature between approximately 50 °C and 100 °C is optimal for fast analyses (77). In fact, it has been demonstrated that heat can substitute for vacuum with not only the 3-NBN matrix, but with 2,5-DHAP (78) and no doubt others, as might be expected, if expansion of subliming matrix or included solvent drives the fracturing process. Using the ASAP method for introducing sample into a Thermo Ion Max source or a Waters Z-spray source and a gentle stream of warm nitrogen gas blown over the area of the melting point tube used to hold the matrix–analyte sample, abundant ions were produced from small molecules to small proteins (Figure 7) (78).

Applications of New Ionization Methods for Mass Spectrometry

The MAIV method is not just applicable to running relatively pure samples, but can be coupled to IMS-MS to provide gas-phase separation and analysis of complex samples. For example, a Waters Synapt G2 system was used to analyze clozapine, levoflaxacin, and cocaine, providing efficient separation of compounds by size, shape, and charge, and high mass-resolution and mass-accuracy as well as MS-MS fragmentation to aid with identification (39). Other classes of compounds can be analyzed such as polymers and additives directly from a few fiber strains taken from the interior of a car (40). Furthermore, by simply spotting the matrix onto a surface, those compounds dissolved into the solvent holding the matrix can be analyzed. Only compounds from the matrix exposed area are ionized (Figure 8). Therefore, by spotting a defect and comparing the results with those from a normal area, it is possible to rapidly determine chemical composition differences in the spotted areas. Because the MAIV method only requires exposing the matrix to the analyte, including surfaces such as biological tissue, or dipping the sample such as tissue piece into the matrix solution, and introducing the matrix-analyte to the vacuum of the mass spectrometer, little user expertise is necessary. Such a simple, yet powerful method has potential in field portable mass spectrometers, and clinical and forensic (Figure 9) (76) analyses.

Although 3-NBN is a powerful MAIV matrix, it does not efficiently ionize all compounds. In fact, basic polar compounds work extremely well with this method whereas nonpolar or acidic compounds are less efficiently ionized. The method seems to have similar attributes to ESI. Although finding a matrix that produces universal ionization is desirable (79), having matrices with selective ionization is also desirable as it allows compounds from a desired class to be analyzed with less interference from undesirable compounds. This is observed when using 3-NBN as a matrix. For example, peptides can be analyzed with very little interference from chemical background, a major interfering component in the low mass range when using MALDI.

The matrices used in MAIV have also been used in conjunction with a DART source (80) and to obtain MS images from surfaces (81), indicating the flexibility and broadness of this new ionization method and this particular matrix. There are no "hot spot" issues with MAIV and because no laser is required, the matrix background, observed at every mass in MALDI below *m/z* 1000 (82,83), is not observed. Because of the continuous ion production, just as in ESI, the reproducibility is improved relative to laser-based ionization technologies, but without using a laser, spatial resolution is decreased. High salt content is usually detrimental to MS analysis, although less so with MALDI than ESI, and seemingly even less so with MAIV where addition of sodium chloride had a rather modest effect with multiply charged protonated ions remaining the most abundant up to a salt concentration nearing 1 M (39).

The newly discovered ionization process for use with MS in which analyte in a solid-state matrix is spontaneously converted to gas-phase ions on exposure to vacuum or modest heat (Figure 1 center and right), is of fundamental scientific interest and has immense analytical potential. The multiply charged ions produced by this method are well suited for high performance mass analyzers with advanced mass measurements in terms of mass resolution and accuracy, ETD structural characterization, and IMS providing inroads into the determination of structure (shape). These capabilities are usually limited to *m/z* <4000, and for ionization methods that produce singly charged ions, larger molecules cannot be analyzed with these instruments. Developing new matrix technology and interfaces designed for the MAIV ionization method will facilitate application to a wide array of problems. However, possibly the most promising

aspect of this new ionization technology is its simplicity and cost savings as the inlet becomes the ion source. A relatively inexpensive mass analyzer with such a simple to use, nearly foolproof, and non-contaminating ionization method might find utility related to human diseases for which current methods are inadequate.

Outlook

As was demonstrated by ESI and MALDI, and, more recently, ambient ionization approaches, successful new ionization methods can have impact on science far beyond anything envisioned in their early discovery. To enhance the rate at which the newly discovered and rather astonishing ionization process contributes to measurement technology, it is of paramount importance to develop a mechanistic understanding of how molecules are transferred from the solid state to the gas phase as ions absent applied energy. This fundamental understanding will have bearing on the mechanism of ionization methods commonly used in MS, guide creation of new matrix compounds, and provide improved methods for high-throughput and spatially resolved analyses. The goal is to advance MS to near real-time molecular characterization of materials maintaining high sensitivity and providing micrometer spatial resolution so that measurements and imaging with high specificity, sensitivity, and dynamic range become possible on a broad range of materials. These goals will be realized by application of knowledge gained from fundamental research to develop a more efficient process for producing and transmitting ions into a mass analyzer from solid surfaces with ever decreasing sampling footprints.

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High-Resolution Native Mass Spectrometry Opens the Door for Detailed Analyses of Intact Protein Complexes

Native mass spectrometry, the method by which noncovalent protein complexes are retained in the gas phase for intact mass analysis, is gaining interest as a method for intact protein characterization. The development of a modified orbital ion trap platform for high-resolution analyses has expanded the role of native mass spectrometry to address the challenges of intact protein characterization. Here, we give an overview of the types of analyses that have recently been accomplished with this new instrumentation, ranging from highly decorated small proteins to large, noncovalent complexes that bind small-molecule ligands. These examples highlight the capabilities of native mass spectrometry and hint at the future role in intact protein characterization.

Natalie J. Thompson and Albert J.R. Heck

The importance of protein analysis has expanded as the role that proteins play in disease progression and potential treatment has become increasingly evident. Many of the diseases we seek to treat stem from phenomena such as misregulation of proteins or misfolding of protein structure. As a way to combat these types of diseases, there is a push for the use of proteins as therapeutics, most notably the development of disease-specific antibodies (1,2). As an example, monoclonal antibodies (mAbs) have been developed to target human epidermal growth factor receptor 2 (HER2) and tumor necrosis factor (TNF) to treat cancers, rheumatoid arthritis, and Crohn's disease. These antibodies number in the top-selling therapeutics (3). To understand these diseases and develop protein-based treatments, it is necessary to delve into protein structure and interactions to construct a detailed network.

Advances in technology have aided in the characterization of proteins and protein networks. The determination of protein primary sequence is currently routine in many laboratories, but it is the characterization of protein–protein interactions and post-translational modifications (PTMs) that remains challenging. PTM analysis is especially crucial because cellular signals are often transduced through PTMs, such as phosphorylation, and PTMs can result from drug degradation (for example, oxidation). Also, for therapeutic antibodies, glycosylation plays a key role in drug clearance and potential immunogenicity. There are several analytical techniques for studying protein interactions and PTMs, such as ion-exchange chromatography (IEC), enzyme-linked immunosorbent assay (ELISA), capillary electrophoresis (CE), and native polyacrylamide gel electrophoresis (PAGE), but mass spectrometry (MS) proteomics methods have become the workhorse for many of these analyses. Often, these interactions and PTMs are detected using enzymatic digestion of a cell lysis or pull-down followed by separation and characterization of the peptides (4). However, this process does not reveal the nature of the intact complexes, and thus these interactions can be hypothesized but not confirmed. Analysis of intact proteins and protein complexes would not only confirm the identity of cellular protein complexes but also provide insight to the multiple proteoforms present (5).

MS has become a crucial technology for the characterization of proteins and protein complexes as advances make it more sensitive and robust. MS was initially relegated to the realm of small-molecule analysis because of the inability to transfer proteins to the gas phase efficiently and effectively. Development of "soft" ionization techniques such as electrospray ionization (ESI) broadened the utility of MS by allowing intact proteins to be detected (6). Though the intact protein was then retained in the gas phase, the protein structure was often lost because of the organic solvents and acids used

as the ionization solution. The development of nano-ESI, which uses a smaller orifice and lower ionization voltages, allowed the incorporation of buffers consisting of aqueous volatile salts, such as ammonium acetate, as ionization solutions, so that aspects of protein higher-order structure and noncovalent interactions could be retained in the gas phase. This native MS (7) is applicable for a wide array of protein samples, with complexes up to 18 MDa (8,9). Recent developments in MS instrumentation have led to the incorporation of native MS conditions on an orbital ion trap platform (10). This combination has yielded high-resolution analyses of proteins and protein complexes, with the ability to characterize complex, heterogeneous PTMs on an intact level. Here, we highlight some recent applications probed using this technology. These examples span the range of size currently achievable as well as emphasize the ability to characterize multiple proteoforms simultaneously present in a single sample. These applications demonstrate the utility of native MS to provide thorough characterization of heterogeneous mixtures and a basis for the future role of native MS for intact protein analysis.

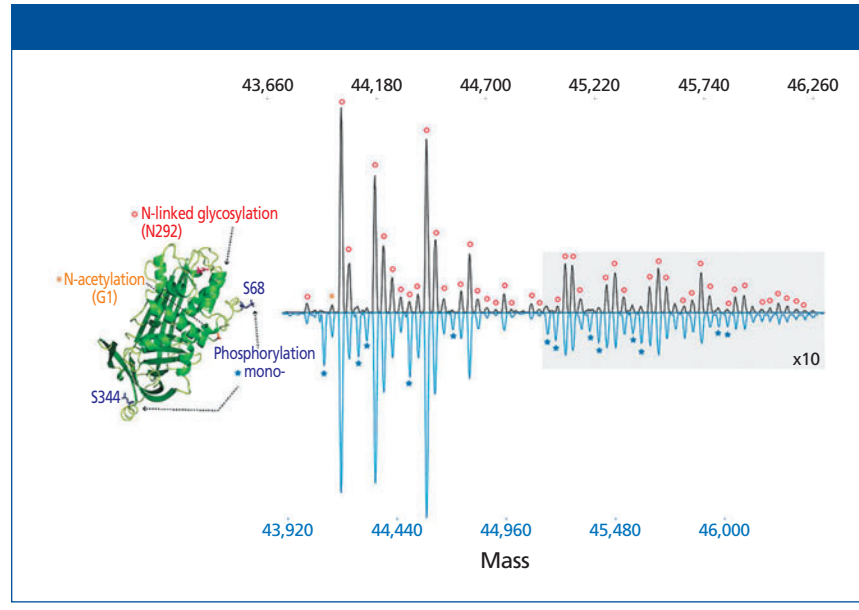

Figure 1: High-resolution native MS identifies 59 proteoforms of intact chicken ovalbumin. The glycan heterogeneity was most easily determined using the dephosphorylated form (top spectrum in black) and each glycan is indicated by a pink circle. Analysis of unprocessed ovalbumin (bottom spectrum in blue) allowed identification of multiple phosphorylation sites, indicated by blue stars. The high mass signals (gray box, multiplied by a factor of 10) are relatively low in abundance yet significantly consist of the less-reported glycan structures. Adapted from reference 18 (copyright ACS Publications).

Methods

Below is a general summary of the sample preparation, instrumentation, and data analysis methods used for native MS of intact proteins and protein complexes. For precise details of these protocols, we direct you to several recent reviews (11,12).

Sample Preparation

A clear advantage of native MS is the simplicity of sample preparation necessary for analysis. The success of native MS hinges on the use of volatile aqueous buffers as solutions for nano-ESI, thus allowing the retention of noncovalent interactions into the gas phase. For the most part, pH-neutral ammonium acetate solutions are used. Protein samples, which are typically purified, are buffer-exchanged using molecular weight cutoff (MWCO) spin filters to ammonium acetate solutions of similar ionic strength and pH as the initial buffer. It is possible to treat the sample before buffer exchange to reduce the complexity of the proteoforms using an enzyme such as a glycosidase or phosphatase. Precise sample treatment depends on the goal of the experiment and will be sample dependent. After buffer exchange, 1–2 µL of a 1–10 µM sample solution is typically introduced to the mass spectrometer under static flow conditions using gold-coated borosilicate capillaries generated in-house.

Instrumentation

Because native MS uses the "gentle" ionization conditions of aqueous volatile buffer and nano-ESI, fewer charges are imparted to the protein ion, thus shifting the charge state distribution to higher *m/z* values. Until recently, this shift meant that time-of-flight (TOF) mass analyzers were required because they were able to provide the mass range necessary for detection. A few instrument modifications are necessary to ensure efficient desolvation, transmission, and detection of these large ions. To assist in desolvation and transmission, the pressure in the first vacuum state is increased (13–15). Other modifications include the incorporation of a high transmission grid and low rep rate pusher in the TOF system, as well as the potential use of a low-rf quadrupole and high-pressure collision cell of tandem MS experiments (16,17).

As mentioned previously, technological developments have made it such that native MS is now possible on an orbital ion trap platform (10). Native MS was initially incorporated on a modified Exactive Plus instrument (Thermo Fisher Scientific). The modifications include manipulations of rf voltages applied to the transfer multipoles to improve ion transmission, software alterations to allow detection of higher *m/z* ions, and manual control of gas composition and pressure in the collision-induced dissociation cell. With these modifications, it is possible to scan from 400 to 30,000 *m/z* and achieve a mass resolution of 25,000 at *m/z* 5000 and 16,000 at *m/z* 10,000, as determined by cesium iodide (CsI) clusters used for mass calibration.

Data Analysis

Deconvolution of the raw data was performed using Protein Deconvolution 2.0 software (Thermo Fisher Scientific). From the zero-charge spectrum, the accurate masses were obtained and the peak intensity was used for any relative quantitation.

Recent Applications

As mentioned previously, native MS is applicable for the analysis of a wide array of intact proteins and protein complexes; however, the achievable mass resolution remained an issue because of inefficient desolvation. The use of native MS on an or-

Table I: Theoretical and experimental values of a 15-antibody mixture. This table lists the values from technical replicates and highlights the small deviations in accuracy and precision for the mass and intensity measurements.

Ab ID	Theoretical Mass (Da)	Experiment 1 Mass	Experiment 1 Intensity (%)	Experiment 2 Mass (Da)	Experiment 2 Intensity (%)	Experiment 3 Mass (Da)	Experiment 3 Intensity (%)	Mass Average (Da)	Mass Error (Da)	Mass Error (ppm)	Intensity Average (%)	Intensity Error (%)
Ab1	144381.84	144382.97	12.09	144382.61	10.06	144383.78	11.99	144383.12	0.60	4.16	11.38	1.14
Ab2	144871.57	144873.45	9.94	144871.84	8.94	144871.95	9.47	144872.42	0.90	6.21	9.45	0.50
Ab3	145361.30	145362.80	5.24	145362.84	4.75	145362.77	4.65	145362.80	0.04	0.27	4.88	0.31
Ab4	145531.24	145530.91	8.21	145532.53	7.09	145531.75	8.11	145531.73	0.81	5.58	7.80	0.62
Ab5	145573.51	145575.50	6.68	145573.55	5.48	145575.00	5.80	145574.68	1.01	6.97	5.99	0.62
Ab6	145673.53	145672.80	8.72	145673.30	7.40	145672.70	7.78	145672.93	0.32	2.19	7.97	0.68
Ab7	146020.97	146022.53	6.82	146022.28	5.76	146021.08	6.27	146021.96	0.78	5.32	6.28	0.53
Ab8	146063.24	146063.59	4.55	146063.67	4.59	146064.25	4.10	146063.84	0.36	2.45	4.42	0.27
Ab9	146163.26	146163.27	7.22	146162.31	6.03	146163.81	6.22	146163.13	0.76	5.19	6.49	0.64
Ab10	146680.65	146680.56	3.84	146680.59	3.02	146680.38	4.09	146680.51	0.12	0.81	3.65	0.56
Ab11	146722.92	146722.63	3.39	146721.78	3.11	146722.02	3.42	146722.14	0.44	2.97	3.31	0.17
Ab12	146765.18	146766.70	3.41	146766.39	2.88	146766.36	2.88	146766.48	0.19	1.30	3.06	0.31
Ab13	146822.93	146821.42	5.18	146822.34	4.33	146821.98	4.84	146821.92	0.46	3.16	4.78	0.43
Ab14	146865.20	146864.83	4.51	146863.42	4.38	146864.38	4.25	146864.21	0.72	4.89	4.38	0.13
Ab15	146965.22	146962.66	5.27	146963.36	4.31	146962.33	4.37	146962.78	0.53	3.59	4.65	0.54

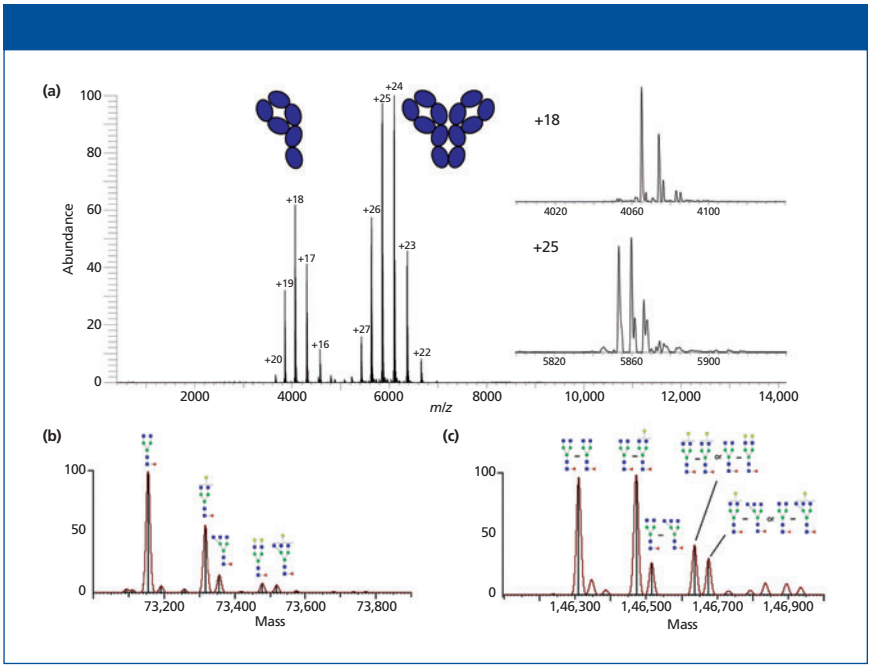

Figure 2: Glycan analysis of intact hingeless IgG4 antibodies. (a) The lack of the covalent disulfide bonds that hold the heavy chains together results in the simultaneous analysis of the half- and whole IgG4 antibody. The high resolution achieved (insets, a) allowed analysis of the glycan heterogeneity (b and c). The deconvoluted spectra show the identification of glycans typically observed for intact antibodies for both (b) the half-body and (c) the whole antibody. Adapted from reference 24 (copyright Landes Bioscience, Inc.).

bital ion trap platform addressed this challenge and, now, highly detailed analyses are possible. Below, we present examples that illustrate the level of detail that is achievable in intact protein analysis.

Defining Protein Microheterogeneity

PTMs play an important role in maintaining the structure and regulating the function of proteins. The detection and analysis of PTMs has progressed with advances in technology, leading to protocols that enrich for PTM-labeled peptides or analysis of released glycans and glycopeptides, for example. Because of the complexity of the microheterogeneity of proteins, PTM analysis of intact proteins remains a challenge. Most of these methods require reduction of sample complexity, often through enzymatic digestion of the protein backbone. In this way, the complete picture of co-occurring PTMs can be lost. The improvements in resolution obtained via the combination of native MS on an orbital ion trap system allow more complete analysis of this microheterogeneity.

The first example to highlight these improvements is the complete characterization of ovalbumin (18). Ovalbumin is a relatively small protein (approximately 45 kDa) that is decorated with an abundance of PTMs, including phosphorylation, glycosylation, and a disulfide bridge (19,20). The combination of these different PTMs has made intact protein analysis a challenge. We took on this challenge and demonstrated that this microheterogeneity could be characterized by native MS (Figure 1) (18). We identified 59 separate proteoforms, and more than half of the proteoforms presented at less than 5% relative abundance. These co-occurring low-abundance proteoforms are often lost using traditional methods.

Complex, extensive glycosylation contributes to the sheer number of proteoforms, and multiple glycans were detected by native MS. Previous analyses of glycans from ovalbumin often attributed unexpected glycans to contaminant proteins (21,22). However, by performing PTM analysis on intact ovalbumin, the observed glycans can be confirmed as bound to ovalbumin, thus eliminating any uncertainty about their origin. The nature of the PTMs was confirmed by selective application of enzymes, such as a glycosidase or phosphatase, demonstrating that intact analysis by native MS was capable of providing a complete overview of the microheterogeneity of ovalbumin.

Aside from complete characterization of a small heterogeneous protein, native MS can also be used to provide an overview of simpler PTMs on larger proteins and protein complexes, such as intact antibodies (mass of approximately 150 kDa). The utility of native MS has been demonstrated for glycan monitoring of half and whole IgG4 antibodies as well as an antibody–drug conjugate (23,24). Because of mutations in the IgG4 antibodies, the hinge was removed, and the two heavy chains were associated solely by noncovalent interactions. And, even though the stability of the antibody structure was reduced, the two heavy chains remained associated in the gas phase and appeared with sufficient resolution as to characterize the attached glycans (Figure 2) (24). In a similar manner, native MS yielded characterization of an antibody–drug conjugate, including both the drug-to-antibody ratio of the different species present and the attached glycans (Figure 3) (24). The use of native MS allowed the retention of noncovalent interactions yielding the intact antibody–drug conjugate even at a drug-to-antibody ratio of 8 (meaning that all disulfide bonds had been disrupted and linked to the cytotoxic drug). Glycoforms could readily be assessed because of clear separation of the signals corresponding to different drug-to-antibody ratios and baseline resolution between adjacent glycoforms. In some of the IgG4 half-antibodies studied, the glycan heterogeneity expanded drastically, with an increase in the branching as well as incorporation of sialic acid. Glycans containing sialic acid are typically a challenge to characterize when released from the protein backbone because of inefficient ionization (25,26). By analyzing the intact protein, the differences in ionization efficiency for the various glycans is reduced and quantitation is possible. This is evidenced through the quantitative comparison of the glycoforms before and after the use of a sialidase to clip the sialic acid from the glycans (24). Removal of sialic acid simplified microheterogeneity of the IgG4 half-antibody and changed the relative abundance of the glycoforms, but the differences in abundance can all be accounted by the simplification glycan structure. These examples emphasize the ability of native MS to retain noncovalent interactions with enough mass resolution that the glycan profiles of intact antibodies are observed, an aspect that could potentially be implemented as a screening technology.

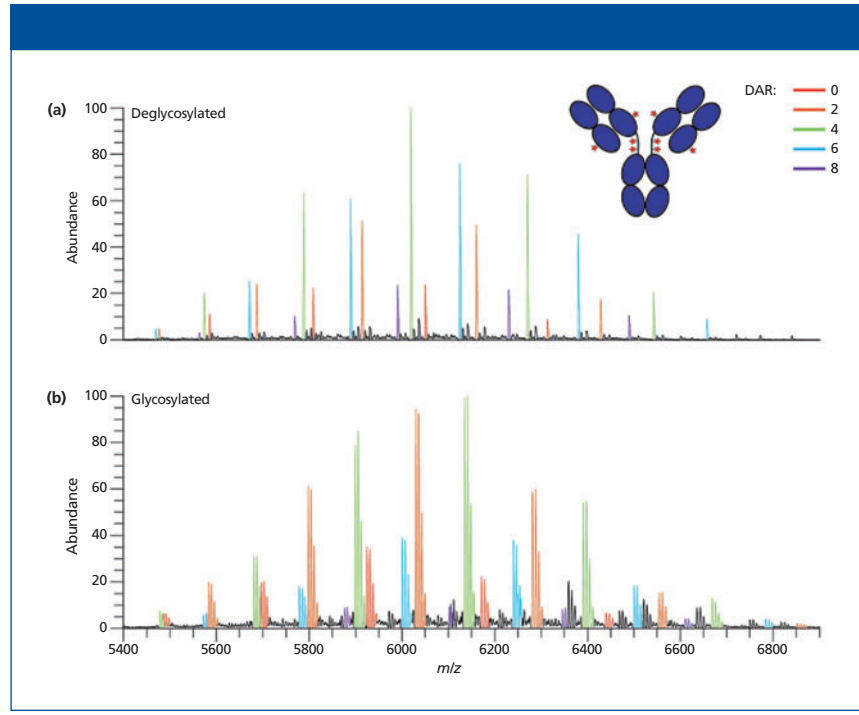

Figure 3: High-resolution native MS yields simultaneous characterization of drug-to-antibody ratio and glycan heterogeneity of antibody–drug conjugate. The commercially available cysteine-linked antibody–drug conjugate brentuximab vedotin (Adcetris) was analyzed in its (a) deglycosylated and (b) glycosylated forms. Adapted from reference 24 (copyright Landes Bioscience, Inc.).

Probing Mixture Composition

The previous examples demonstrate how native MS on an orbital ion trap platform can provide a detailed overview of the variety of PTMs that can be present on an intact purified protein. However, some samples are more complicated such as antibodies engineered to work as a mixture. As the level of bioengineering increases for antibody-based therapeutics, the analytical methods must also advance to tackle the challenges of characterizing a mixture of highly related components. For example, the production of antibody–drug conjugates often results in a heterogeneous mixture because of different drug payloads on the protein backbone. This is evident by the presence of multiple species differing by drug-to-antibody ratio (Figure 3) (24). Another example is the recent interest in using a combination of antibodies as a single therapeutic (2,27,28). Currently, these therapeutics consist of individual antibodies that are characterized individually and administered simultaneously (29). For this simple mixture, the analytical characterization methods are already established, but as mixtures become more complex, the cost of time and resources for the production of these mixtures grows to the point of being prohibitive. To address this future issue, there is a push for novel technology to produce mixtures of antibodies on a single platform (29–31). With these advances in mixture generation, analytical characterization becomes more difficult. In 2012, we compared native MS with cation-exchange chromatography for the analysis of these types of antibody mixtures and found that

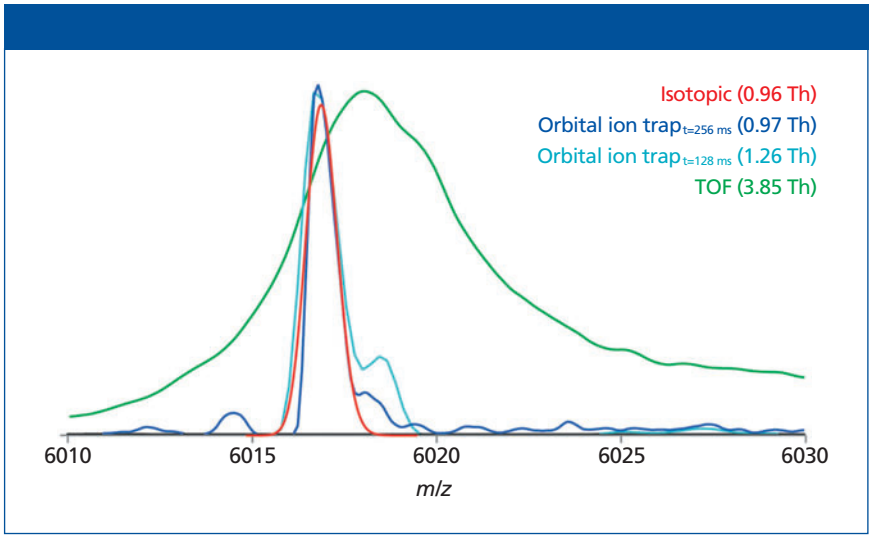

Figure 4: Experimental data approaches theoretical peak width. The overlap of raw native MS signals for a single IgG1 antibody is shown with that of the theoretical signal. The peak widths at half-maximum abundance (FWHM) is shown in parentheses. Adapted from reference 33 (copyright Landes Bioscience, Inc.)

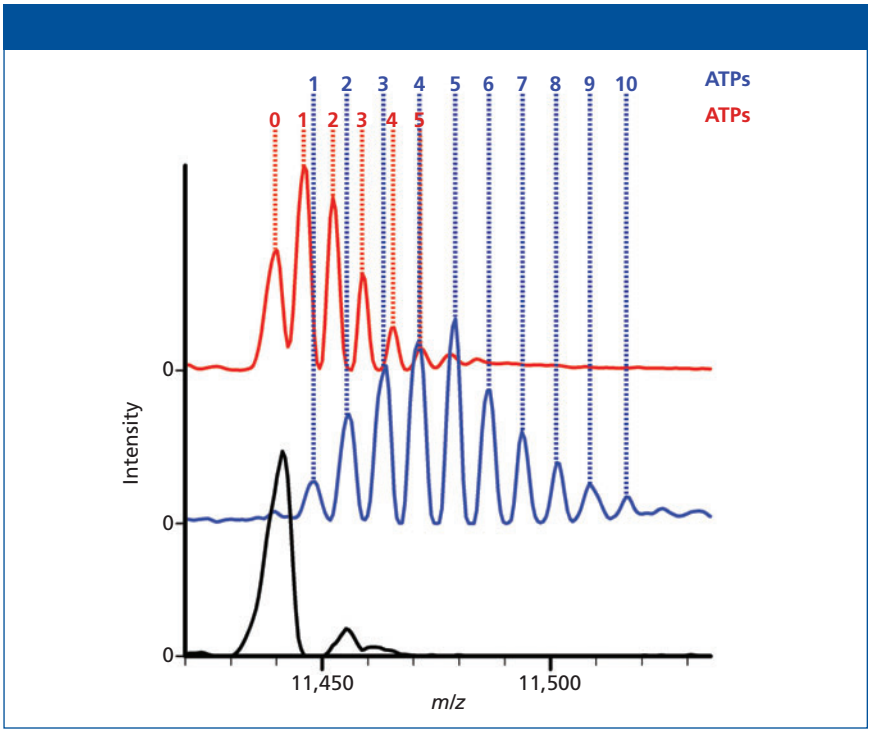

Figure 5: Native MS on the orbital ion trap platform allows investigation of small mass changes to high mass complexes. Detection of small molecule ligands binding to large intact protein complexes is illustrated by the binding of ADP and ATP to intact GroEL (801 kDa). The mass spectra show unbound GroEL (black) and that bound to ADP (red) or ATP (blue). The baseline resolution between adjacent species allows the counting of individual molecules, as indicated by the numbers. Adapted from reference 10 (copyright Nature Publishing Group).

native MS yielded comparable quantitation with the advantage of identification and quantitation in a single experiment (32). We also noted that native MS is able to handle increasing mixture complexity better than cation-exchange chromatography. The increased resolution obtained via the orbital ion trap platform only expands the utility of native MS for mixture characterization. We recently probed the limits of this technique by characterizing mixtures containing 6, 10, and 15 antibodies (33). We found that baseline resolution was achieved with mass differences as little as 42.27 Da for intact, deglycosylated antibodies. A comparison of peak widths revealed that experimental resolution approaches that of the theoretical isotopic peak width resulting in supreme mass accuracy (Figure 4) (33). This mass accuracy, an average of 7 ppm, is crucial for consistent identification and confirming the primary sequence and PTMs. The mass accuracy, combined with high mass and quantitative precision, less than 7.5 ppm and 1.2%, respectively (Table I), forms the basis of a powerful method for comprehensive mixture characterization.

Obtaining Details for Protein Complexes

Analysis of single proteins is necessary for probing the complex microheterogeneity that is often present, but proteins rarely exist individually. Instead, protein complexes and the interactions of various subunits regulate functions and pathways. In the initial study of native MS on an orbital ion trap platform, a wide range of protein complexes was studied, spanning from intact antibodies (150 kDa) to the molecular chaperone GroEL (801 kDa) (10). Not only was baseline charge-state resolution achieved, but the addition of small molecules could be monitored. For example, incubation of GroEL with ADP or ATP resulted in the sequential binding of these small ligands to monomers in the 14mer protein complex. The addition of ADP or ATP molecules to intact GroEL was easily resolved (Figure 5), equating to additions of less than 0.1% total mass (10).

Discussion and Future Outlook

The drive for development of novel biotherapeutics also pushes the limits of traditional analytical technologies leading to the concurrent development of comprehensive new methods. Native MS has been gaining interest as a result of its many applications, like those described above. The combination of native MS on an orbital ion trap platform has expanded its utility to now be able to provide a detailed overview of the microheterogeneity of intact proteins and protein complexes. Several other advantages make native MS a valuable asset for biotherapeutic characterization. Inherent

to MS-based techniques, very little sample is required (on the order of femtomoles) and, unique to native MS, uses a straightforward, limited sample preparation without worrying about the reproducibility of proteolytic digestion. The rapid acquisition time means that results are obtained in minutes compared to hours and copurified contaminant proteins are often evident because of drastic differences in mass. However, these analyses do require specialized instrumentation that is often modified to transmit and detect these large ions (see above). While native MS provides a comprehensive overview of protein microheterogeneity, exact localization and structure of the PTMs, such as the connectivity and branching of a glycan, cannot be determined. Also, several PTMs result in moderate to large differences in mass, but there are those like deamidation that are much more readily detected by cation-exchange chromatography than native MS.

The high resolution afforded by the combination of native MS on an orbital ion trap platform can provide the detailed analysis of intact proteins that may be used as a high-resolution fingerprint for analytical characterization of biotherapeutics. Native MS has the ability to identify various components in a mixture, whether they be PTM-based microheterogeneity or highly related antibodies in a composite mixture, as well as quantitate these components in a single experiment. The retention of noncovalent interactions and tertiary structure allows the creation of a complete picture of the intact protein and its proteoforms as well as expanding the applicability to protein complexes. Improvements in automation, both in sampling (34,35) and data analysis (36,37), are leading to simplifying the implementation of this technology in the laboratory. In our opinion, the benefits of high-resolution native MS would be readily realized in the characterization of complex biotherapeutics and can provide insight to the microheterogeneity of protein structure and function.

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Identifying "Known Unknowns" in Commercial Products by Mass Spectrometry

The identification of nontargeted species in environmental and commercial samples by mass spectrometry can be very difficult. In this article, authors from Eastman Chemical Company describe their systematic approach for the identification of nontargeted species using nominal and accurate mass data, searching both mass spectral and "spectra-less" databases.

James L. Little, Curtis D. Cleven, and Adam S. Howard

Organic mass spectrometry (MS) has witnessed an extraordinary increase in capabilities this past decade because of major advances in ionization sources, analyzers, detectors, chromatography, and computer technology. Many of these technological advances focus on biological applications, a fact plainly evident to attendees of the American Society for Mass Spectrometry's (ASMS) annual conferences. Yet the significance of this ever-sophisticated technology has not been lost on industrial, environmental, and forensic mass spectrometrists, whose work involves characterizing commercial chemical products.

Eastman Chemical Company is a global manufacturer of polymers, fibers, coatings, additives, solvents, adhesives, and many other products. Gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) have proven to be essential for characterizing our company's products and those of other companies. With reasonable effort, we routinely and reliably obtain mass spectral data from these highly sensitive and yet robust techniques. However, unless the data can be converted into structural information, it is not useful as a knowledge base to resolve the analytical problem at hand.

In the last 34 years, we developed and refined a systematic process (1,2) for the identification of nontargeted species using GC–MS and LC–MS analyses. We refer to these types of species as "known unknowns" — that is, species known in the chemical literature or MS reference databases, but unknown to the investigator. The essence of the process is finding candidate structures by searching mass spectral databases, Chemical Abstract Services databases, and ChemSpider databases. Figure 1 presents a simplified flowchart of the overall process; the subsequent sections discuss individual steps and illustrate three examples in the identification of known unknowns.

Computer-Searchable Mass Spectral Databases

The first step in the process is computer searching of spectra against mass spectral databases. This approach (3) is very powerful and efficient for the identification of unknowns typically requiring 3–5 s for each component in a mixture. Electron ionization (EI) databases are used for identifying compounds in GC–MS analyses, and collision-induced dissociation (CID) databases are used for LC–MS analyses. The databases are purchased from commercial sources or are created from compounds characterized at our company (see Table I).

The results of the EI mass spectral searches are normally more successful than CID searches for two reasons. First, the number of entries in EI databases for GC–MS is approximately 10 times larger than that for CID databases for LC–MS. Second, 70-eV EI spectra are much more reproducible than CID spectra, which can vary significantly depending on instrument design and user-specified variables (3).

NIST MS Search Software as Eastman Corporate Standard

We adopted the National Institute of Standards and Technology (NIST) MS Search program as our corporate standard for searching mass spectral databases for the following reasons:

- Searches both EI and CID databases
- Performs fast EI searches with essentially no false negatives (3)
- Searches libraries by spectra, structure, and other data fields
- Merges search results for multiple databases
- Creates users' libraries with structures and other data fields
- Merges, archives, and distributes users' libraries nightly
- Imports spectra and structures from all major commercial software programs
- Correlates fragments to substructures for EI and CID spectra via MS Interpreter utility

The automated process of merging, archiving, and distributing our corporate EI and CID databases occurs nightly by means of batch files and a simple event-scheduler utility. A standard GC–MS laboratory computer on the network serves as the sole library server for our company, which operates a worldwide computer

network of MS systems. Many of these remote systems are operated by scientists with minimal expertise in mass spectral interpretation. When necessary, those scientists send their files via the network for interpretation by corporate experts in MS. The experts then add spectra and associated structures to our corporate database.

Soft Ionization for Molecular Weight Determinations

The molecular weight of a component is one of the most important pieces of information obtained from MS analysis. CID spectra obtained by LC–MS analyses that use "soft" ionization techniques, such as electrospray ionization (ESI) and atmospheric-pressure chemical ionization (APCI), normally yield ion species that indicate the molecular weights of components. In contrast, the molecular ions of components often go unobserved in EI analyses. We use chemical ionization (CI) to determine the molecular weights of those components in EI GC–MS analyses (4).

We use a wide variety of CI gases and gaseous mixtures in GC–MS analyses including methane, isobutane, ammonia, ammonia-d_3 (5), methylamine, and others. The choice of gas depends on the proton affinity of the unknown. We primarily use ammonia, however, because most of our unknowns contain heteroatoms. Ammonia CI yields very good molecular weight information (proton adducts, ammonium adducts, or both). Moreover, it does not leave carbon deposits that contaminate and ultimately hinder the performance of the CI source. MS CI manifolds supplied by the manufacturers for many of our GC–MS instruments are incompatible with ammonia gas, so we fit our instruments with custom manifolds (5). In addition to tolerating ammonia, the custom manifolds provide easy in situ preparation of gaseous mixtures.

Accurate Mass Data for Molecular Formula Determinations

The wide availability of time-of-flight (TOF), quadrupole TOF (QTOF), and orbital trap mass analyzers allow the routine acquisition of high resolution mass spectral data with low parts-per-million (ppm) mass accuracy in either LC–MS or GC–MS modes. In many cases (6), even a mass accuracy of <1 ppm is inadequate to determine a unique molecular formula (MF). Therefore, mass spectrometry vendors apply orthogonal filters such as isotopic ratio abundances and a variety of heuristic and chemistry rules (7) to limit the number of molecular formulas.

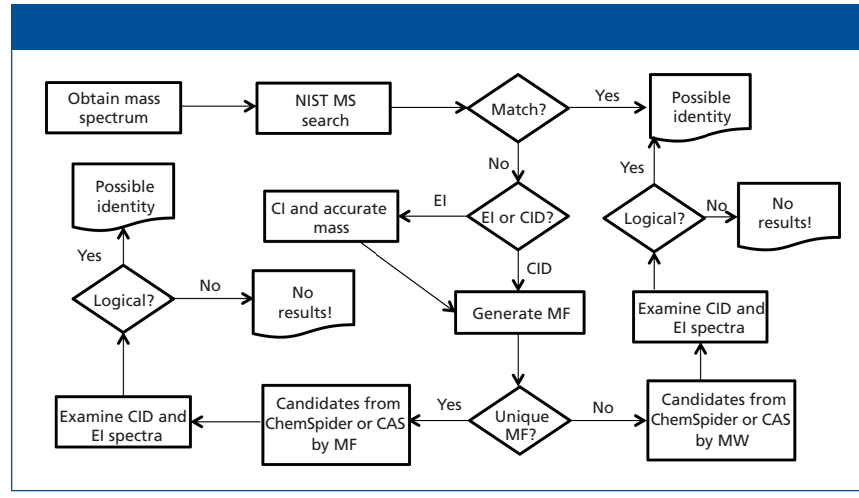

Figure 1: Simplified flowchart for identifying "known unknowns." MF = molecular formula and MW = molecular weight.

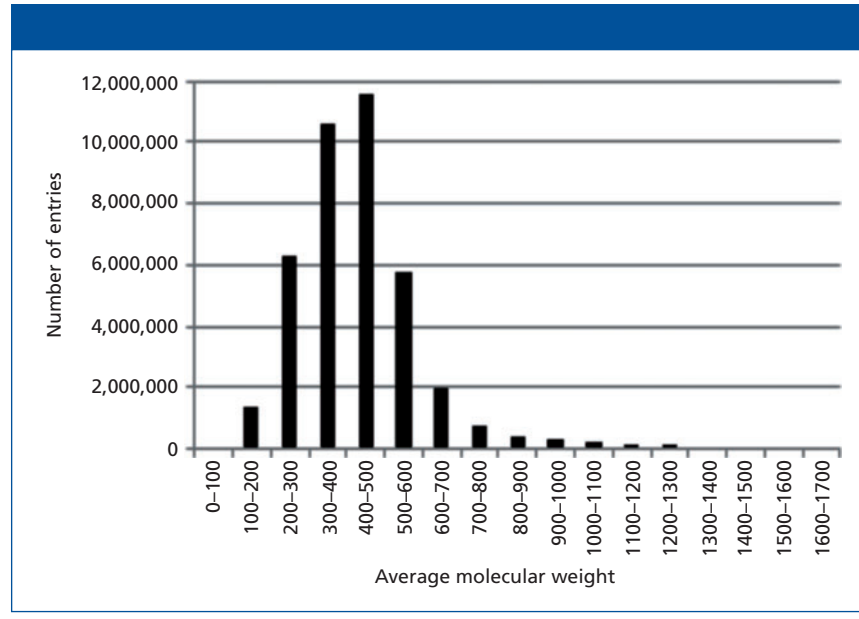

Figure 2: Number of CAS registry substances versus average molecular weight.

Searching "Spectra-Less" Databases

The limited number of spectra in commercial databases as well as our corporate mass spectral databases dictates the use of other databases for the identification of known unknowns. For many years, we have used "spectra-less" databases (8,9) such as our internal plant material database and the Toxic Substances Control Act (TSCA) listing for identifications. These databases contain no computer searchable mass spectra and are searched only by monoisotopic mass, average molecular weight, or molecular formula. Unfortunately, data evaluation proved tedious because no orthogonal filters were available to prioritize the candidate lists, and these databases did not offer structures.

Recently, we have found the Chemical Abstracts Service (CAS) Registry (>70 million substances) and ChemSpider (>28 million entries) are particularly valuable spectra-less databases for identifying known unknowns (1,2). Both databases are accessed via intuitive web-based interfaces. The CAS Registry is a fee-based system and ChemSpider is provided as a free resource

Table I: Spectra with associated structures searched with NIST search software

Source	EI Spectra (structures)	CID Spectra (structures)
NIST	243,893 (212,538)	82,616 (82,334)
Wiley	662,860 (559,014)	12,048 (11,806)
Eastman	54,986 (28,229)	3856 (3405)
Total	961,739 (799,781)	98,520 (97,545)

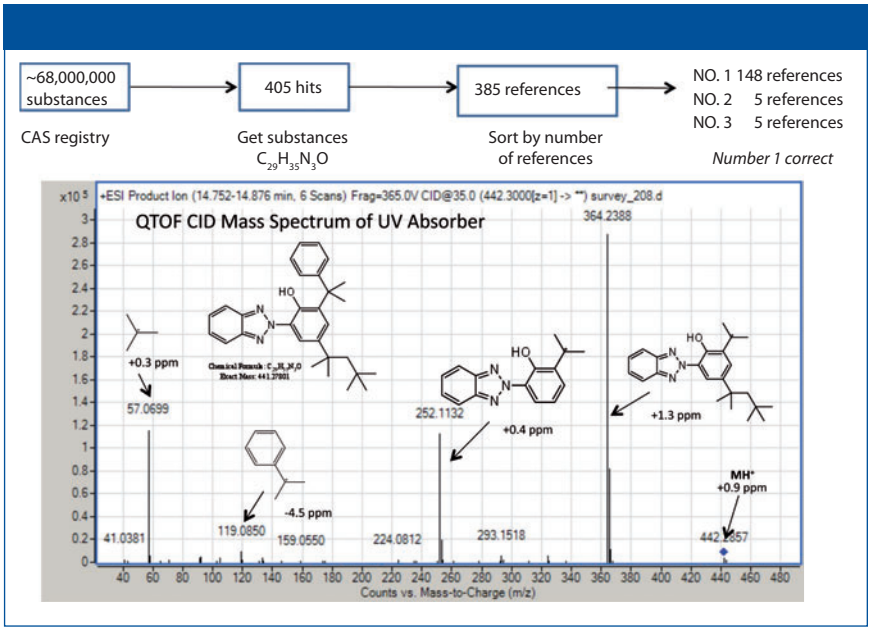

Figure 3: Confirmation of a polymer additive identification by the major fragment ions noted in its accurate mass CID spectrum obtained in positive ion mode.

to the community. Both are searched by molecular formula and the results are sorted by the number of associated references. The correct structure routinely appears among the top 1–5 hits in the sorted lists for a variety of classes of compounds.

The highly curated CAS Registry includes many key words associated with substances. For relatively obscure substances (that is, those with fewer associated references), it is often very useful to query the list of candidate compounds by key words determined from minimal sample history (1). This approach is not available within ChemSpider.

Searching Spectra-Less Databases by Molecular Formula Vs. Molecular Weight

The molecular formula is without doubt the best search parameter for querying spectra-less databases for candidate structures. However, we demonstrated (1,2) that searching by molecular weights can be particularly useful when unique molecular formulas cannot be determined for higher molecular weight (>600 Da) compounds. In theory, as the molecular weight of an unknown increases, the number of possible molecular formulas increases dramatically. Yet in practice, the number of compounds observed in both the CAS Registry (Figure 2) and ChemSpider databases decreases dramatically as a function of increasing molecular weight.

You can search the ChemSpider database by a monoisotopic mass using a mass range consistent with the accuracy of the user's measurement. In contrast, you can only search the CAS Registry with SciFinder by average molecular weight, to the hundredths decimal place. Monoisotopic mass can be determined much more accurately than average molecular weight (1,2), thus the former is the preferred approach. Nevertheless, even with relatively large windows of 70 ppm, known unknowns can be routinely identified searching the CAS Registry by average molecular weight. Thereafter, the candidate list from the average molecular weight query can be further refined using monoisotopic mass and isotopic abundances as well as the number of associated references and key words.

Narrowing the Search of Databases to an Exact Structure

All candidate lists obtained by searching spectral or spectra-less databases are further refined, if possible, to a unique structure. Refinements of the lists are performed using a wide variety of additional ancillary information (1,2). This information includes EI spectra, CID spectra, sample history, the number of exchangeable protons (1,2,5), UV–vis diode-array spectra, nuclear magnetic resonance (NMR) data, types of ion adducts, relative retention times, the presence of related compounds in sample mixture, chemical derivatization, hydrolysis (10), and more.

Of this information, the most critical factor is the EI or CID spectrum of the known unknown. We often obtain both EI and CID spectra for an unknown because we find the GC–MS and LC–MS data yield complementary information. In many cases, the sample might require derivatization to form the trimethylsilyl derivative (11) for GC–MS analyses. The spectra are interpreted manually using model compound spectra obtained via the NIST similarity structure search of available reference databases. The NIST MS Interpreter utility correlates observed ions with molecular substructures for the model compounds as well as for the unknown and its associated putative structure.

Of course, not all components of a mixture are identified using our approach. Finding "no results" (Figure 1) can occur for many reasons. Some compounds have relatively few associated references, or the appropriate keyword to properly prioritize the candidate list cannot be determined. Also, some compounds can convert in the sample matrix to "unknown unknowns." These "transformation products" (12) are not found in any spectral or spectra-less databases. In either case, we often succeed in identifying these additional compounds by correlating similar fragment ions and neutral losses of the unknowns to other components identified in the mixture.

Purchasing a standard of the material for comparison is, ultimately, the best means of confirming the identity of a known unknown. Both ChemSpi-

der and SciFinder are particularly useful for finding commercial sources of standards. Also, identifications are routinely confirmed by using microreactions and known chemistry to prepare a mixture enriched in the compound of interest.

Examples That Illustrate the Process

The following section presents three typical examples of our approach for identifying known unknowns using spectra-less databases. No examples were included for the library search of EI and CID spectra because that approach is relatively straightforward. You can find many additional examples in our previous work, which includes detailed screen captures for SciFinder and ChemSpider queries (1,2).

Example 1

An additive was noted in a commercial polymer. The additive's molecular formula, as determined by accurate mass, LC–MS, and ESI data, was $C_{29}H_{35}N_3O$. Results from a SciFinder search of the CAS Registry by molecular formula were sorted by the number of associated references and the top hit was CAS No. 73936-91-1, a UV absorber. The identification was confirmed by the major fragment ions noted in its accurate mass CID spectrum in positive ion mode (see Figure 3).

Several other pieces of data supported this identification. The compound was shown by infusion ESI analysis to have one exchangeable proton. We routinely confirm exchangeable protons by both ND_3 CI and ESI analyses (1,2,5). Furthermore, the UV–vis diode-array spectrum revealed the expected absorbances, 302 and 342 nm, for a UV absorber. The identity in this case was confirmed with data from a purchased reference standard.

Example 2

An unknown was noted in the extract of a can coating. Polymers used in food contact applications must meet strict criteria for extractables when appropriate food-simulating solvents are used. A polyester coating was applied to metal cans at a contract laboratory and the expected linear and cyclic polyester oligomers were observed in the extracts. In addition, an unexpected UV-absorbing species was noted with a molecular formula of $C_{36}H_{40}O_6$.

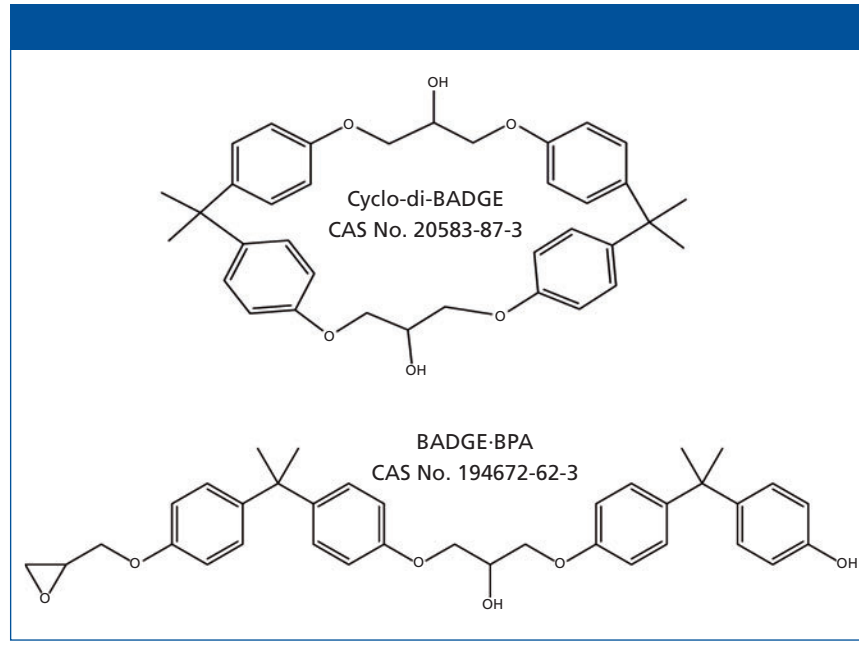

Figure 4: Structures of cyclo-di-BADGE and BADGE·BPA.

The references associated with the candidate list from SciFinder were further refined. "Can coating" was specified as a keyword, and the two likely isomeric structures shown in Figure 4 were found.

The negative ion electrospray data were consistent with the cyclo-di-BADGE (di-bisphenol A diglycidyl ether) isomer. BADGE·BPA yields both $[M-H]^-$ and $[M + acetate]^-$ anions, whereas cyclo-di-BADGE yields only the $[M + acetate]^-$ anion because the latter species contains no phenolic end group. The identification was confirmed by extracting a BADGE can coating and by preparation of BADGE·BPA from the reaction of BADGE with bisphenol A.

Cyclo-di-BADGE is a common, low-molecular-weight, cyclic monomer noted in the extracts from epoxy-based resins used in can coatings (13). The contract laboratory had inadvertently contaminated our coating with the material.

Example 3

An additive, whose monoisotopic mass was determined to be 783.520 ± 15 ppm, was observed in a polypropylene polymer. The top hit in the search of the ChemSpider database by monoisotopic mass yielded CAS No. 27676-62-6, an antioxidant, as the top candidate in the list when sorted by the "Number of References" field (see Figure 5).

The proposed identity was confirmed by accurate mass, CID fragmentation, which yielded the major ion at *m/z* 219 and three exchangeable protons by ESI infusion of the mixture. The supporting data were so convincing that the identification was immediately reported to the customer. At a later date, a standard of the material confirmed the initial identification.

Future Enhancements

Our current process for identifying known unknowns in simple mixtures is very useful and reasonably efficient. However, it is too time-consuming for complex mixtures. Although efficient, searches of EI and CID databases are significantly limited by the reporting process. We are working with several companies to resolve this limitation.

The searching and reporting of data from spectra-less databases is much more complicated and time-consuming. The main bottleneck in the process is the manual interpretation of CID spectra. We have done some initial work using a "systematic bond-breaking" (14) approach that automatically scores the observed fragment ions in a CID spectrum to computer-generated fragment ions for candidate structures. Thus, a group of candidate structures are automatically exported from ChemSpider to Agilent's prototype version of the Molecular Structure

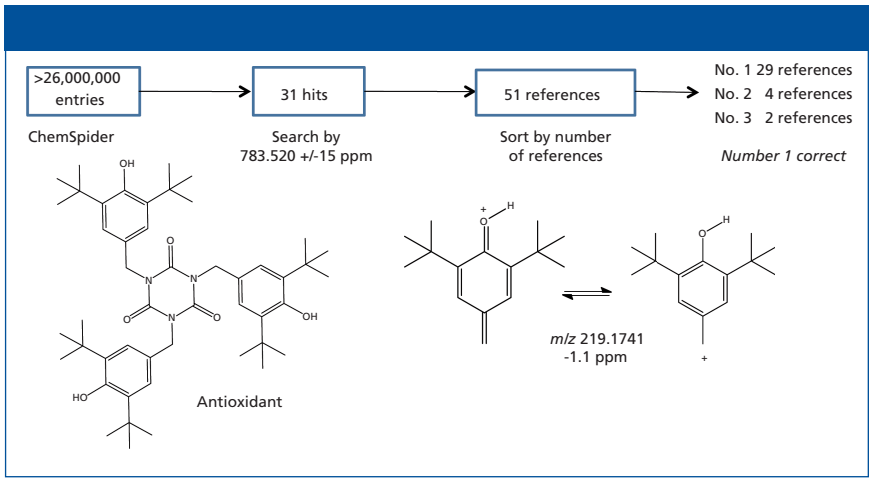

Figure 5: Identification of an antioxidant in polypropylene polymer.

Correlator program (15). The program sorts the results by the number of references, assigns a score based on observed ions versus computer-generated ions, and displays substructures associated with accurate mass fragment ions. It would be useful if SciFinder would utilize a similar application program interface.

Finally, many chemical structures prove difficult to predict manually. For others, it is difficult to generate fragment ions using a computer. Therefore, it would be useful if the actual fragmentation (fragments and neutral loses) from model compounds in reference CID databases could somehow be correlated with the candidate structure.

Conclusions

Eastman Chemical Company has developed a systematic process for successfully identifying compounds in commercial products using a variety of approaches. The easiest approach uses a computer search of EI and CID spectra using the NIST MS Search program. The software, provided free of charge with the NIST EI and CID reference databases, enables us to create, archive, and automatically distribute user databases to our worldwide corporate network.

The other approach uses web-based searches of spectra-less databases, such as ChemSpider and the CAS Registry, by molecular formula or molecular weight. The candidates are assigned priority according to the number of associated references or keywords. The top hits are then refined by their EI or CID spectra and many other orthogonal filters.

Supplementary Information

Supplementary information is available upon request from the author including library searching, library networking, additional examples of SciFinder and ChemSpider searches, selection of chemical ionization gases, silylation reactions and associated artifacts, polyester analyses, matrix ionization effects, and surfactant identifications.

Acknowledgments

Regrettably, it is not practical to individually recognize all those who have contributed to our approaches for identifying known unknowns in the last 34 years, though many are mentioned as authors in the references and associated acknowledgments. We will, however, acknowledge two individuals whose influence on this work is most eminent. The initial concepts originated from collaborations with Bill Tindall of Eastman Chemical Company. Steve Stein, of NIST, and his talented staff developed the software and utilities. CAS Registry Number is a registered trademark of the American Chemical Society.

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The Fundamental Shift to Tandem Mass Spectrometry

In this article, we examine how tandem and tandem hybrid mass spectrometry has opened up new frontiers already. We go further and examine how lesser-known experiments are breaking new ground, with alternative fragmentation techniques, as well as the addition of extra levels of orthogonality by parallel separations techniques.

St. John Skilton, Eric Johansen, and Xu Guo

Today, in the biopharmaceutical industry mass spectrometry (MS) is a critically useful and efficient tool for routine and investigational analysis in therapeutic discovery, development, and production. Almost every analytical department now routinely uses MS at some stage in the process of therapeutic development.

However, there is one intriguing aspect that is somewhat surprising given the prevalence of MS; that is, a monolithic view held by some whereby all mass spectrometers or all techniques are lumped into a broad category labeled "MS." This is all the more surprising given that the experiments performed are enormously varied. It is the view of these authors that such convenient shorthand results from a predominance of a small number of MS experiment types adopted by the industry. Although many may know that alternative experiments exist, few have the time to explore them and many may be unaware of the extreme utility of these experiments for greater efficiency and information, with little time penalty or method development. In this article, we touch on how tandem mass spectrometry (MS-MS) has developed and how alternative uses of it may better inform the industry and speed up therapeutic design and development, with particular reference to the biopharmaceutical area.

A Brief View of History

The development of MS-MS has not been seen as obvious, and has relied partly on fortuitous results and typical scientific curiosity about fundamental gas phase reactions (1,2). In the 1970s the use of MS-MS was extremely informative about the behavior of ions in the gas phase and their dissociation, although it remained highly academic (3,4). In experiments that often used enormous magnetic sector instruments, advanced research was still looking intently at what was later termed "fundamentals," reflecting how the field was aiming to understand the very mechanisms of what was occurring (5). In fact, MS-MS research had been a steady thread of activity right from the very start of mass spectrometry, beginning more than a century ago (1). However, the 1970s saw the massive rise of a plethora of instrument types, including some ambitious multiple-sector instrumentation. One type was the tandem quadrupole, which opened up what has arguably been the most commercially successful type of mass spectrometer ever invented, and which still dominates the market today (8). In common terminology, this has become known as a *triple quadrupole*, on the basis that the middle quadrupole segment was the collision cell, although this mechanism has long been superseded.

But here too lies one of the continuing puzzles for many people in the field: Why has the variety of experiment types not been used more widely? The most predominant MS-MS experiment remains that used for quantification of analytes: *multiple reaction monitoring* (MRM), whereby a precursor is selected, and a small subset of the fragments are subsequently monitored to determine very precisely how much of the analyte is present — mostly with reference to isotopically labeled standard analog species. However, almost all tandem mass spectrometers have the inherent capability of looking "backward" by using the fragment ion species to reconstruct what the precursor molecule was like. This has been extensively explored in the metabolite identification world — for example, where predictable biotransformations can be mapped by integrating the MS and MS-MS information with informatics packages (10). Additionally, it is also possible to look backward and mark out the parts of a molecule that are not present because they didn't ionize or were broken into pieces that are not recognizable. Examples of this are "constant neutral loss" experiments, or "parent–precursor ion scans" (13). It is all the more surprising that these types of experiments are not per-

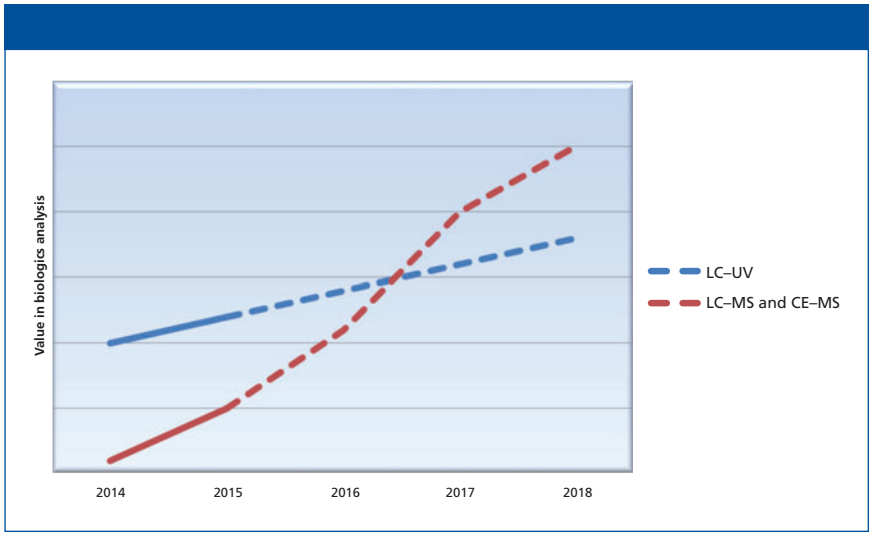

Figure 1: Graph showing the estimated relative adoption rates of MS-based detection versus optical detection for the biopharmaceutical market (2014–2018). Both techniques grow above 5% per annum, but MS-based techniques accelerate as more biotherapeutics reach the market and pipeline. (Data sources: various, including FiercePharma, PhRMA reports, public company reports; collated by the author.)

formed more frequently because they can be done almost simultaneously in certain types of mass spectrometers (for example, tandem quadrupoles and quadrupole time-of-flight [QTOF] systems).

Tandem in Space and Time

Here too, the subtle differences in what tandem mass spectrometers actually are is instructive: tandem itself is a conflation of "tandem in space" and "tandem in time." The examples all mentioned above are of the tandem-in-space type, whereby the instrument components that analyze each of the successive fragments are separated physically. This is not the case for instruments that are "trapping," in which ions can be stored for some period of time, potentially forever. This distinction is important, because the differences in tandem types have largely determined which types of instruments have been sold for specific applications in the pharmaceutical market. Traps that store specific ions and subject them to even more analysis are extremely good at homing in on one specific thing, and providing an extremely in-depth view of what that analyte is — even though it may be at the expense of other molecules present simultaneously. On the other hand, tandem-in-space instruments are extremely well suited for looking at a number of things simultaneously, perhaps at the expense of losing detail on specific items. So, why not have a combination of both attributes? In many ways, this is partly where the instrumentation industry has headed, with some surprisingly creative results.

Economically Viable Adoption

By the time the tandem quadrupole was being widely adopted for clinical work in the mid 2000s, it was already profiting from rapid advances, so the invention of what many have described as a new "class" of instrument was also well accepted (9). But additionally, by this stage many researchers were working directly with the nascent biopharmaceutical industry and were extremely oriented toward the actual samples that needed to be analyzed. In an example of this, Hopfgartner and colleagues (9) commented:

> ". . . the uniqueness of the instrument is that the same mass analyzer Q3 can be run in two different modes [quantitative and qualitative] . . . [one mode,] EMC . . . offers obvious advantages, in particular for samples containing very low peptide levels. For many analytical challenges, selectivity often becomes more important than sensitivity."

In that article, Hopfgartner and colleagues also compared the capabilities of trapping with "axial" ejection, which coped with the limited storage capacity of three-dimensional (3D) traps. The limited storage capacity can be a hindrance when a mixture of large, highly charged species are examined together, where the ions that are more predisposed to become highly charged may force the exclusion of lower abundance species from the spectrum. In instruments with "linear" ion traps, it is one of the reasons why low relative abundance is not as problematic (9,13). The capability to sequence peptides of low abundance was noted.

Perhaps two of the answers as to why more MS-MS experiment types are not used are profit reasons and the relative slowness in the rise of truly intuitive software. The profit aspect relates to economic imperatives that determine whether instruments are able to provide value for money — and of course in constrained economic times this is all the more apparent. These authors suggest that as we move forward, greater emphasis will be placed on the biopharmaceutical industry obtaining tools that are able to simultaneously provide extensive experiment types — productivity — as well as allow post-experiment investigation — security. Now that many experiment types are instantaneous within the constraints of typical experimentation, the ability to go back to find data that may have always been there becomes much more feasible. In fact, many of these advances have been quietly adopted as standard tools, including the ability to automatically assign peptide sequences and look at the sequence simultaneously (14). The use of informatics has progressed,

Figure 2: (a) An example of an automatically assigned peptide map by LC–MS-MS where each of the peaks is labeled by software. The panels at the bottom indicate the orthogonal evidence available to the reviewer in the event of queries. The coverage achieved was 98% over the 60-min run. (b) An example of an automatically assigned peptide map of the molecule trastuzumab by capillary electrophoresis electrospray ionization (CESI) separation with a color-coded assignment of the peptides identified (100% coverage).

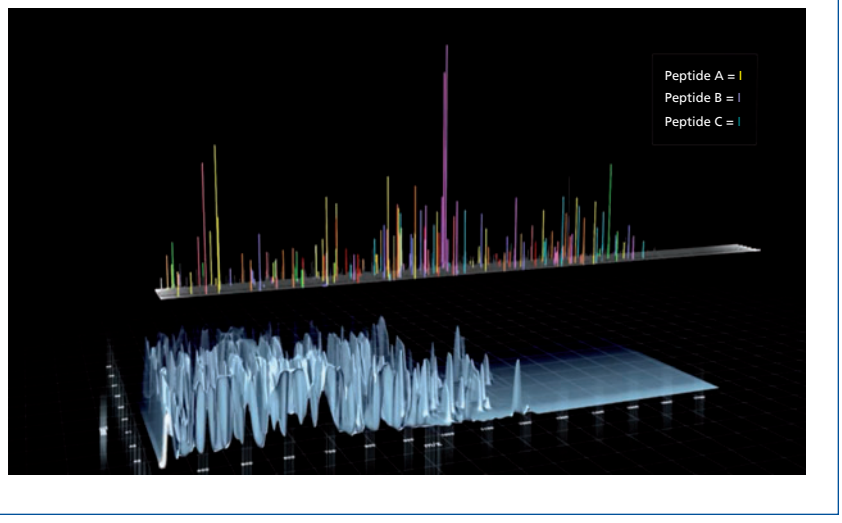

Figure 3: Graphical illustration of data acquisition using informatics software (Swath, AB Sciex) where precursor and fragment ions are collected through an entire experiment. This technique provides a comprehensive dataset in a peptide map that can be remined at a later date with new hypotheses or to extract information that was not predicted. For more information see reference 16.

but perhaps by virtue of not being as emotionally exciting as advances in expensive capital equipment, it is paradoxically perceived as less exciting. But there has been a crucial development in the industry: the ability to go back and re-examine data with fresh hypotheses without new sample injections. So, the combination of instrument capability and informatics has provided some real benefits, not least of which is the automation of many tedious tasks otherwise performed by humans.

Conclusion

The practical application of MS-MS has long been recognized, and a subset of the experiments has long been a default tool in the industry (MRMs). But it is reasonable to argue that the depth of capability has remained untouched. It can be argued that it is economic imperatives that push organizations to start to use more of the tools already at their disposal to obtain more out of their capital expenditures. In some cases, this might mean finding contaminant host cell proteins more rapidly, or identifying previously unknown sequence variants in an effort to improve product quality as quickly as possible (15). Under all circumstances, MS-MS will only increase in value, usage, and, understanding.

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Information Rich Flash Chromatography II: All-Wavelength Collection and Purity Measurement

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UV-visible detectors have been used on flash chromatography systems for several years to control the fraction collector. The purity measurements were limited to ratio measurements that required *a priori* knowledge of the spectra of the compound and impurities so that the correct wavelengths could be used both to collect peaks and determine purity.

All-wavelength collection is a technique that monitors all detector wavelengths in a user-defined range. A change of absorbance within that range is treated as another peak and triggers collection or peak cutting by the fraction collector. All-wavelength collection can also be employed to determine peak purity over the entire spectral range chosen by the chemist with real-time solvent background suppression.

Background

All-wavelength collection is a technique useful for purifying compounds when the UV spectrum is unknown, as is the case for natural products. When measuring the UV spectra of synthesized mixtures, the UV of the desired compound may be masked by the stronger spectra of impurities causing an inappropriate detection wavelength to be chosen. All-wavelength collection helps to ensure the desired compounds are collected, even in the presence of solvent-induced baseline changes. When compounds closely elute, it is useful to be able to resolve the mixture based on peak purity measurements. Measurement of the entire UV spectrum in all-wavelength collection allows the possibility of purifying compounds by purity index measurements.

Experimental and Results

Experiments were run on a Teledyne Isco Combi*Flash* Rf+ PurIon system. Solvents were ACS grade from VWR Scientific (Radnor, Pennsylvania).

Example: Initial Purification of Green Tea Compounds

A t1 g extract was dissolved in methanol and adsorbed onto Celite 545 (Acros Organics) in a RediSep® solid load sample cartridge (PN 69-3873-235). The compound was eluted with a hexane:isopropanol gradient followed by an isopropanol:water gradient on a 15.5 g RediSep Rf gold diol column (PN 69-2203-515). The alkaloids and catechin compounds eluted together while the tannins eluted early in the water gradient. Fractions were collected using the all-wavelength collection algorithm.

A diol column was used because a single gradient run captures nearly all the compounds ranging from very non-polar through water soluble.

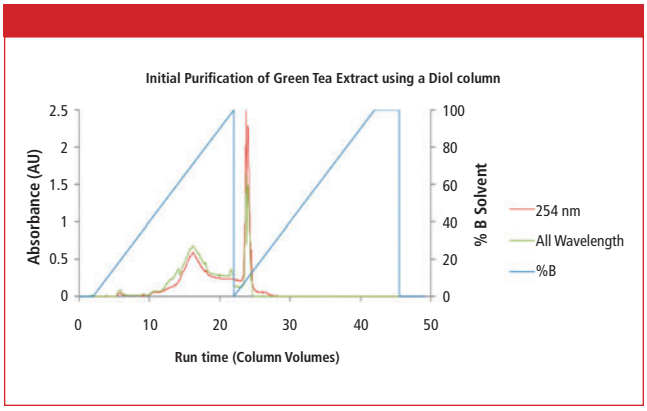

Figure 1: Initial purification of green tea extract with all-wavelength collection and a diol column.

Fractions were collected and combined from 10–21 column volumes (CV) based on TLC and comparisons to authentic samples. The peaks at 22 CV are probably tannins. These were found to adsorb irreversibly onto polyamide. The all-wavelength collection and trace compare favorably to that of detection at 254 nm.

Conclusion

All-wavelength collection can be used to detect and fractionate compounds when their absorbance isn't known. All-wavelength collection can compensate for a drifting baseline such as that induced by solvent absorbance. Compounds and sample loadings that generate peaks that saturate a UV detector at a given wavelength can also be fractionated with all-wavelength collection.

A full version of this application note can be viewed at: www.isco.com/WebProductFiles/Applications/101/Application_Notes/AN94_Information%20Rich%20Flash%20Chromatography%20II.pdf.

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Utilization of CESI Technology for Comprehensive Characterization of Biologics

Rajeswari Lakshmanan, PhD, AB Sciex

Monoclonal antibodies (mAbs) form a major class of biologics and recently biosimilars and biobetters are being added to the growing inventory of therapeutics. In-depth characterization of mAbs at various stages of development and manufacturing is essential to maintain product safety and efficacy. However, analysis of mAbs is challenging due to their high molecular weight, the microheterogeneity presented by the glycans, and degradative modifications that occur during production. Any analytical technique that provides greater depth of information without a time penalty is an advantage. A recent advancement to meet this need was the introduction of CESI–MS. CESI is the integration of capillary electrophoresis (CE) and electrospray ionization (ESI) in a dynamic process, within the same device. In this technology, the analytes are separated inside an open nontapered capillary based on their electrophoretic mobility, and electrosprayed directly into the MS (2). At operating flow rates less than 30 nL/min, very efficient desolvation and, thus, ionization is achieved.

Though high speed CESI separations reduce analysis time, it also necessitates the use of high speed MS to preserve the separation efficiency. The TripleTOF® 5600+ system (AB Sciex) offers the necessary high acquisition speed, high resolution, and high mass accuracy, in both MS and MS-MS modes. CESI performance was evaluated by analyzing a tryptic digest of trastuzumab using the SCIEX CESI 8000 - TripleTOF® 5600+ MS platform.

Experimental Conditions

Trastuzumab was reduced, alkylated, and digested with trypsin. After drying, it was resuspended in the leading electrolyte (100 mM ammonium acetate at pH 4) and 50 nL (100 fmol) was injected into the separation capillary. The background electrolyte used was 10% acetic acid and a separation voltage of 20 kV (normal polarity) was applied for 60 min. Information dependent acquisition (IDA) was utilized to trigger MS-MS. IDA parameters were optimized so that the duty cycle of the MS was matched to the high speed CE separation. Data analysis was performed using BioPharmaView™ software (AB Sciex, Massachusetts).

Results and Discussion

Primary Sequence Coverage: 100% primary sequence coverage of both the light and heavy chains of the antibody were obtained. Peptides ranging from 4 to 63 amino acids in length without any missed cleavages were detected. Electrophoretic separation is based on the charge-to-mass ratio of the peptides and is not dependent on relative hydrophobicity. Thus, small hydrophilic peptides often lost in the LC void volume, and large hydrophobic peptides, which tend to be retained on the column, can be identified by CESI–MS, resulting in the high sequence coverage observed.

PTM Characterization: Data from CESI-MS analysis showed the presence of several PTM hotspots such as N-terminal pyro-Glu formation, methionine oxidation, and asparagine deamidation. Pyroglutamination leads to loss of a positive charge which results in the electrophoretic mobility of the modified peptide being lower than the unmodified one. This is advantageous since the modified and unmodified forms can be separated by CESI–MS and the MS-MS spectra confirmed the presence of the pyro-Glutamate residue (Figure 1). Oxidative degradations at Met^{255} and Met^{431} and deamidations at Asn^{55} and Asn^{387} in the heavy chain and at Asn^{30} in the light chain were also identified. A typical identification from the CESI–MS data is shown in Figure 2.

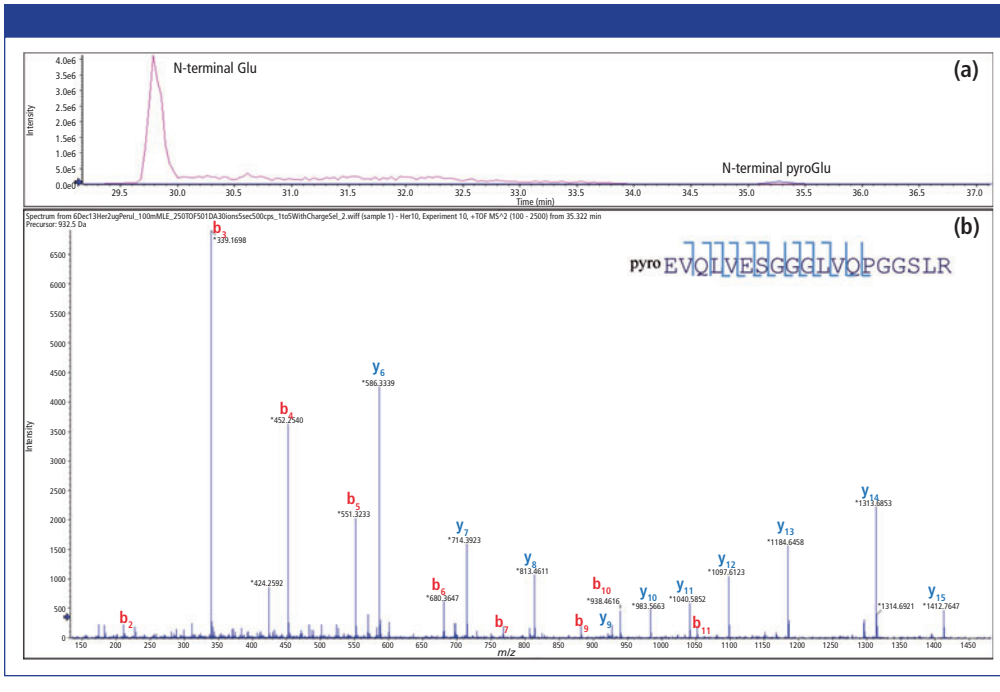

Figure 1: (a) Extracted ion electropherogram of N-terminal peptide with Glu and pyroGlu separated by CESI and (b) MS-MS identification of N-terminal peptide with pyroGlu.

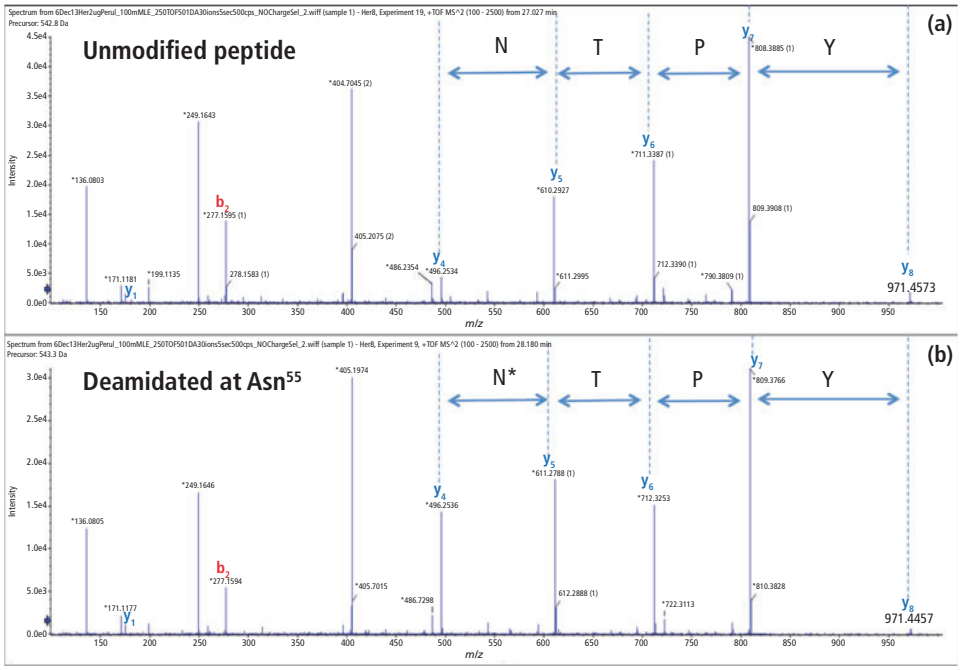

Figure 2: MS-MS identification of asparagine deamidation with (a) showing unmodified peptide and (b) deamidation at Asn^{55} in the heavy chain of trastuzumab.

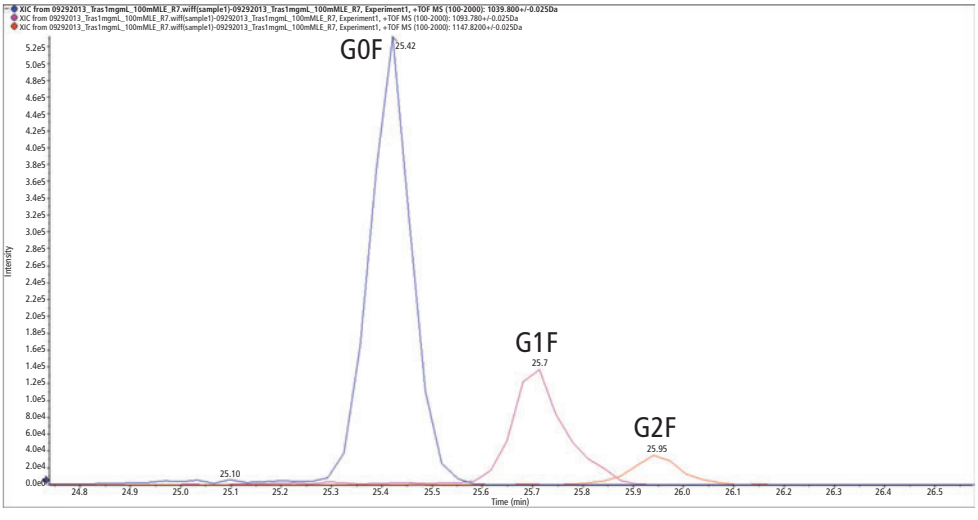

Figure 3: Extracted ion electropherograms of peptide TKPREEQYNSTYR with G0F, G1F, and G2F modifications.

Glycosylation Heterogeneity: Trastuzumab possesses one N-glycosylation site at Asn^{300} in the HC where different glycoforms such as a-fucosylated or fucosylated glycans can be present (3). By using CESI-MS, the G0F, G1F, and G2F forms of the peptide TKPREEQYNSTYR were separated well as shown in Figure 3. Furthermore, the identification of G0F, G1F, and G2F forms of peptide EEQYNSTYR without the missed cleavage (at the arginine residue in the peptide TKPREEQYNSTYR) also confirmed the presence of these glycoforms. In addition, the a-fucosylated forms of this peptide, such as G0 and G1, were identified, but it has to be further confirmed that the a-fucosylated forms were not generated due to source fragmentation of fucosylated counterparts.

Conclusions

We have presented CESI–MS, a robust ultra-low flow and highly efficient separation technology in combination with TripleTOF MS, a high resolution accurate mass measurement system for qualitative analysis of biopharmaceuticals. CESI–MS is attractive for simultaneous analysis of primary sequence coverage and glycopeptide profiling, without carry-over concerns. The combination of high separation efficiency and high sensitivity allows the analysis of all peptides including modified and low abundant species, in addition to confirming the amino acid sequence of the antibody.

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Arsenic Speciation by HPLC–ICP-MS with High-Sensitivity Ion Focusing and Nitrogen-Enriched Plasma

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The effects of nitrogen addition to an argon plasma for ICP-MS analysis have been extensively studied over many years in numerous applications for elemental analysis (1–3), but without resulting in widespread commercial or regulatory acceptance. Recent research at Bruker (4) combined with simple integration of this technique into current and existing hardware shows some renewed promise for this application, with potential for significant cost-benefit advantage. The effects of this enhanced ionization and resulting sensitivity gain will be demonstrated, to show potential benefits of N_2-enriched argon plasma on selectivity and detection limits for As chromatography, using high-sensitivity quadrupole ICP-MS. The results will be evaluated against and in conjunction with more traditional collision and reaction interference removal technology (5).

Enhancing the ionization of transition metals such as As and Se in argon plasma spectroscopy has been studied extensively over decades. While the mechanisms involved are not well understood, the effects of altering the plasma conditions vs. matrix additives have been well characterized.

The increased sensitivity of arsenic combined with the interference removal using CRI technology is demonstrated in Figure 1. N_2 addition to the plasma improves As response under all conditions, however the optimal conditions are N_2 addition combined with H_2 reaction gas to give both the most effective background removal and the largest difference between blank and a calibration standard. Based on these results, a similar enhancement in As ionization and improvement in detection limits is expected for chromatographic measurements.

The As^+ signal enhancement comes in addition to the already high sensitivity of the aurora Elite ICPMS, which is capable of ion count rates >1.5E+6 c/s/ppb under normal plasma conditions, using Bruker's patented ion mirror technology. This enables unparalleled ion focusing efficiency while effectively removing residual gas from the ion beam and eliminating photons as a source of background noise, due to the dual-off-axis spectrometer design.

Equipment

The aurora Elite high-performance ICPMS with CRI technology; Advance UHPLC+OLE solvent delivery system; Bruker-CTC

Figure 2: Schematic view of Bruker's ion mirror and off-axis quadrupole fringe rods.

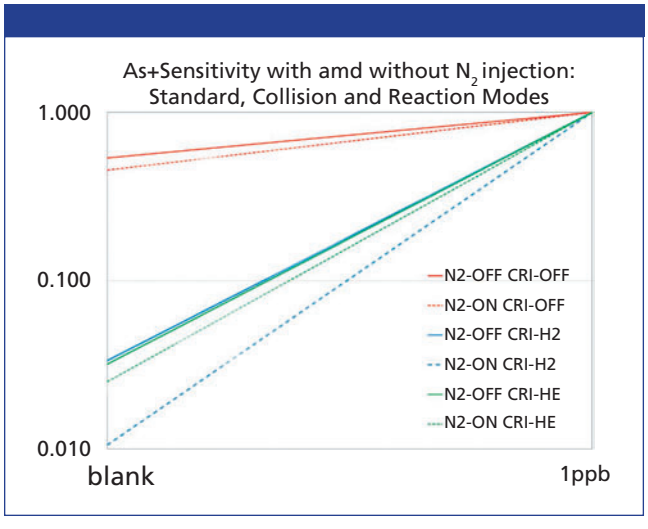

Figure 1: Relative signal-to-noise of As^+ measurements under varying conditions of collision and reaction interference removal coupled with N_2 injection into plasma in a 1%HNO_3/HCL matrix.

Figure 3: The Bruker aurora Elite – Advance µHPLC–ICP-MS system.

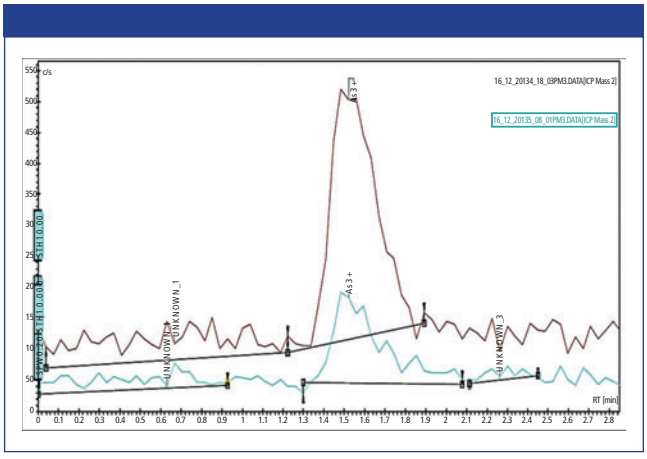

Figure 4: Comparison of 0.025 ppb As^{3+} with (red) and without (blue) N_2 addition to plasma.

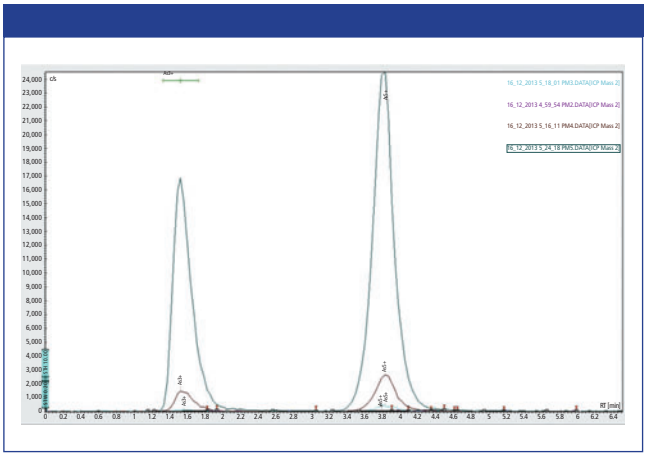

Figure 5: Overlay of multiple chromatograms; 0.0, 0.025, 0.25, and 2.5 ppb As^{3+} and As^{5+}, without N_2 addition.

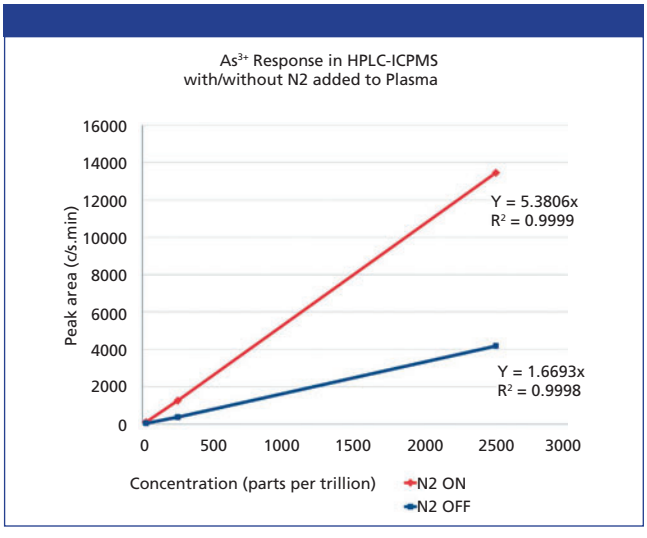

Figure 6: Comparison of peak areas and linear response calculations for multiple As^{3+} calibration standards.

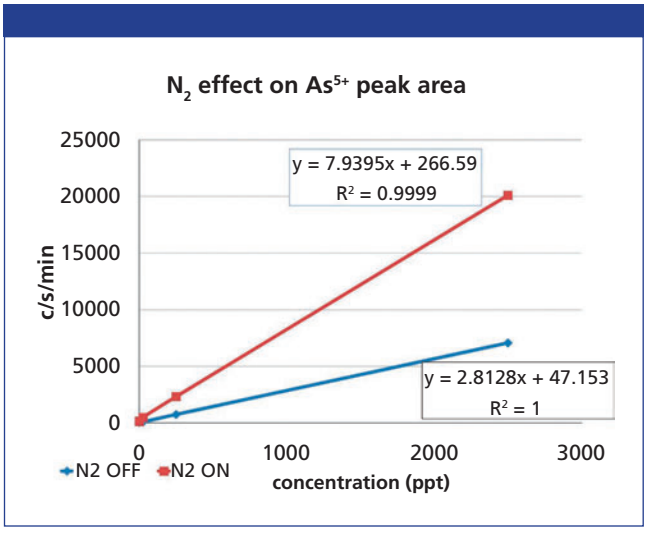

Figure 7: Slope and linearity calculations for As^{5+} response with and without N_2 addition.

PALxt DLW autosampler; CompassCDS software for complete system control and data management

Results

By keeping all other instrument operating parameter constant, the enhancement effect of N_2 addition on the As^+ response was clearly observed, even at concentrations <50 ppt.

Conclusion

N_2 addition to the argon plasma is a simple yet effective technique for improving the response for As (and Se, not shown here) in elemental speciation analysis. Better sensitivity and reduced background will result in lower detection limits and improved analytical capabilities.

Future Work

Improvements to the chromatography should yield even better results — significant blank contamination was discovered during the present work. Similar results for Se speciation and for more complex organometallic compounds (for example, arsenobetaine, and monomethylarsenate) will also be shown. The positive effects of N_2 addition to the plasma on other molecular ion species formation (particularly ArO^+) has also been observed, and this will be investigated for improving Fe+ response in biochemical analysis such as SEC. As can be seen in Figures 4–7, the result of N_2 addition to the plasma has a significant effect on As^+ signal response and resultant sensitivity, regardless of the ionic state of As^+.

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Bruker Daltonics Inc.
40 Manning Road, Billerica, MA 01821
Website: www.bruker.com

PRODUCTS & RESOURCES

Mass spectrometer

The LCMS-8050 triple-quadrupole mass spectrometer from Shimadzu is designed to perform both positive and negative ion analysis in a single run. According to the company, the spectrometer provides multiple reaction monitoring speeds of 555 ch/s and maintains spectrum quality, sensitivity, and accuracy at scan speeds of up to 30,000 u/s.

Shimadzu Scientific Instruments, Columbia, MD; www.ssi.shimadzu.com

Sampler with centrifuge

Gerstel's CF 200 centrifuge is designed for use with the company's MultiPurpose Sampler and reportedly can perform centrifugation of up to six samples at up to 4500 rpm (2000*g*). According to the company, centrifugation power can be specified in revolutions per minute or in times gravity, and replaceable rotors for 2-mL or 10-mL vials are available.

Gerstel GmbH & Co., KG, Linthicum, MD; www.gerstel.com

Flash chromatography system

Teledyne Isco's CombiFlash Rf+ PurIon flash chromatography system is designed to identify a peak of interest at the time of elution with targeted fraction collection. According to the company, the system is supplied with its PeakTrak software, which allows access to commands from a single touchscreen.

Teledyne Isco, Lincoln, NE; www.isco.com

LC–MS system

The Orbitrap Fusion Tribrid LC–MS system from Thermo Fisher Scientific combines quadrupole, Orbitrap, and linear ion-trap mass analyzers and is designed for the analysis of complex biological samples. According to the company, users can choose between fragmentation modes at any stage of MS^n analysis and unknowns in small-molecule experiments can be identified conclusively as they are encountered.

Thermo Fisher Scientific, San Jose, CA; www.thermoscientific.com

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