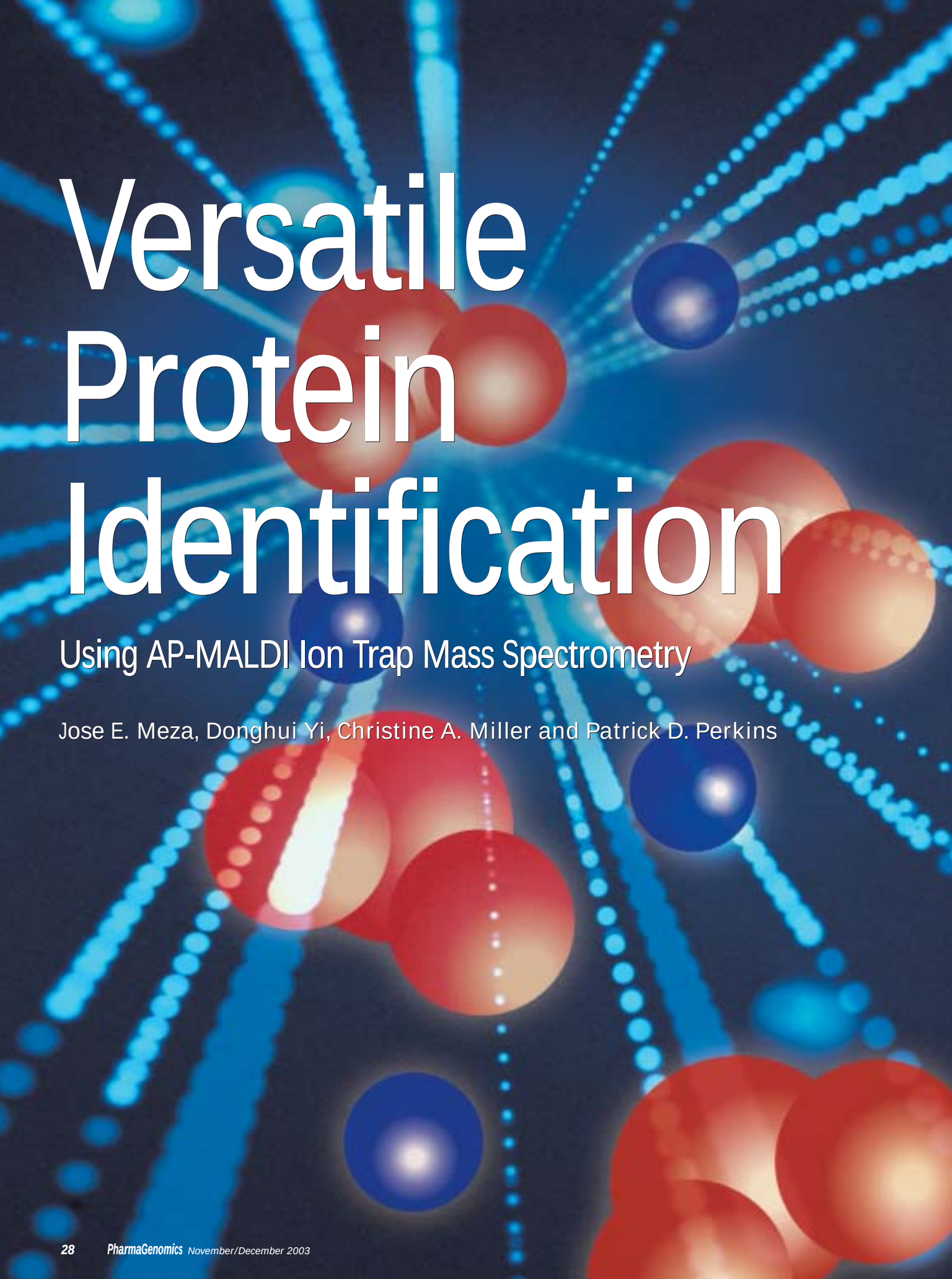


Versatile Protein Identification

Using AP-MALDI Ion Trap Mass Spectrometry

Jose E. Meza, Donghui Yi, Christine A. Miller and Patrick D. Perkins

BACKGROUND PHOTO: IMAGE 100

Q3DM

New developments in matrix-assisted laser desorption ionization (MALDI) technology enable decoupling of the ionization source from the time-of-flight mass analyzer for operation at atmospheric pressure (AP-MALDI). This technique also can be integrated with other types of mass analyzers, such as the ion trap. This article describes the use of an ion trap mass analyzer that supports interchangeable AP-MALDI and electrospray ionization (ESI) sources, a platform that enables selection of the MS technique best suited to the experimental objective and facilitates acquisition of complementary AP-MALDI and ESI MS-MS data.

Historically, two-dimensional gel electrophoresis (2-D GE) in conjunction with matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been the instrument platform of choice for proteomic analysis. A 2-D GE/MALDI-TOF assay of a tissue sample typically can yield from 1000 to 2000 proteins ranging across six orders of magnitude in abundance (1). After separation, protein samples can be analyzed directly to determine their molecular weight and also can be subject to enzymatic digestion, followed by MS analysis. The resulting collection of mass data constitutes a "peptide mass fingerprint" that can be used to identify a precursor protein by matching it against entries in a protein sequence database. Advantages of MALDI-TOF MS include the speed of setting up and carrying out analyses, as well as a large mass range and high detection sensitivity.

Despite being applicable to a large subset of the human proteome, 2-D GE methods are limited when used to analyze certain classes of proteins. These limitations include difficulty analyzing membrane and other hydrophobic proteins, highly acidic and highly basic proteins and, in particular, low-abundance/low-molecular-weight proteins (2). This latter class includes many proteins involved in cellular functions, such as signal transduction, kinase pathway regulation and control of transcription events, and is of growing interest to investigators seeking potential targets for therapeutic intervention. Attempts to isolate and digest low-abundance proteins for subsequent MS analysis can be confounded easily by inadequate sample recovery. Two-dimensional GE methods are not fully automated, and the requirement for manual sample handling can introduce unacceptable losses. Given the fact that the analytes of interest are low-abundance proteins, losses can cause analyte concentrations — and even more so the concentration of their respective digest peptides — to fall below the method's limit of detection. Furthermore, low-abundance proteins isolated by 2-D GE could be masked by higher-abun-

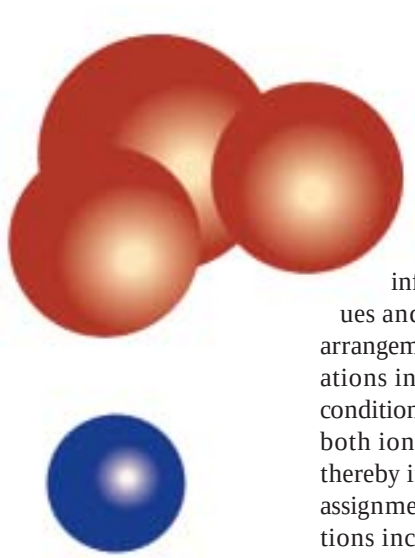
Table I. Comparison of MALDI-TOF MS and MALDI Ion Trap MS

Conventional MALDI-TOF	AP-MALDI Ion Trap
Well-suited for protein/peptide analysis	Well-suited for protein-digest/peptide analysis; also can be used for small molecules and polymers
Soft ionization – structural information requires complicated and expensive Q-TOF or TOF-TOF instruments	Soft ionization – easily acquired MS-MS information from more cost effective ion trap
Dedicated instrument	One instrument for both ESI and MALDI
Laser intensity affects mass resolution and accuracy	Laser intensity does not affect mass resolution or accuracy
Positioning of sample plate holder critical for mass accuracy	Positioning of sample plate is less critical
Vacuum system requires sample pump-down; requires frequent calibration	Ionization takes place at atmospheric pressure; infrequent calibration — shorter analysis time; higher sample throughput

dance proteins overlapping the same gel location and can go undetected.

An additional problem involves the difficulty of carrying out precursor ion selection for MS-MS or, in the case of MALDI-TOF MS, comparable post-source decay experiments (3, 4). Because the analytes of interest already are at a very low concentration, the inability to acquire adequate sequence information could constitute an insurmountable obstacle to utilizing this technique in protein identification, not to mention in *de novo* sequencing.

The lack of an easy way to acquire MS-MS data is not the only drawback associated with MALDI-TOF MS. The tight coupling of the ionization process to the mass spectrometer is a source of systematic errors. In MALDI, the spread of velocities in the laser-ejected ion plume determines the arrival time of ions of differing mass at the detector. Given proper system calibration, this information is used to calculate mass values and abundances. The problem with this arrangement lies in the fact that even slight variations in system parameters (as well as other conditions affecting ion generation) can perturb both ion flight times and spatial separations, thereby introducing mass resolution and mass assignment errors. Examples of such perturbations include inconsistent matrix deposition, variation in spot dimensions or changes in laser focus, pulse energy or duration; even slight changes in MALDI stage or laser positioning can have an effect. As a result, MALDI-TOF instruments require continual optimization to compensate for these deviations. Taken together, the problems associated with MALDI-TOF MS have created an interest in MALDI MS configurations that are both truly MS-MS capable and more robust.



Two-Dimensional LC–ESI MS and MS-MS

To overcome the methodological and instrumental limitations associated with 2-D GE/MALDI-TOF MS, researchers and instrument manufacturers have focused on the development of alternative nanoscale analytical platforms for proteomics. One approach that has received attention, and subsequent utilization, combines two-dimensional liquid chromatography (2-D LC) separations with electrospray ionization (ESI) ion trap MS (2). These systems address problems of limited sample size, low-abundance analytes and poor recovery by completely automating sample handling and transfer operations, incorporating capabilities for sample concentration and providing mass detection sensitivities now in the range of 100 amol per µL or better. The ion trap mass spectrometer's inherent MS-MS capability meshes well with ESI, which produces multiply-charged peptide ions that are well suited for generating sequence information via MS-MS fragmentation. While the nanoscale 2-D LC–ESI ion trap MS system does provide an instrument platform capable of addressing the analytical requirements for low-abundance/low-molecular weight analytes, there is a significant tradeoff in throughput. Long analysis times sometimes are required to resolve and characterize the very large number of sample components produced in complex multi-protein digest mixtures. A sample consisting of an enzymatic digest of only several proteins can contain hundreds to thousands of peptides and

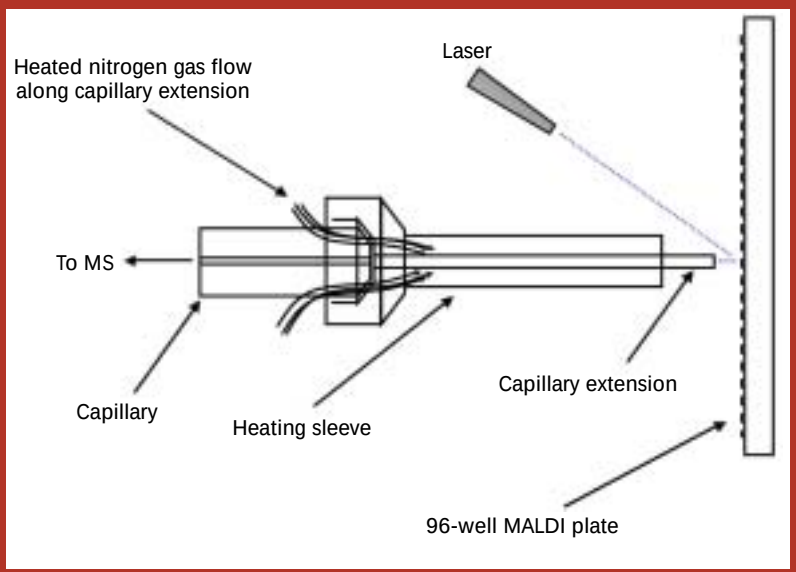


Figure 1. Schematic of the AP-MALDI source. Sample and co-crystallized matrix are desorbed from the 96-spot target plate using a pulsed 10-Hz nitrogen laser beam (337 nm) incident at an approximately 45° angle to the plate. Resultant gas-phase ions are drawn into the capillary extension, while admission of low-mass matrix clusters is minimized by using a heated nitrogen gas flow around the capillary extension, countercurrent to the ion beam.

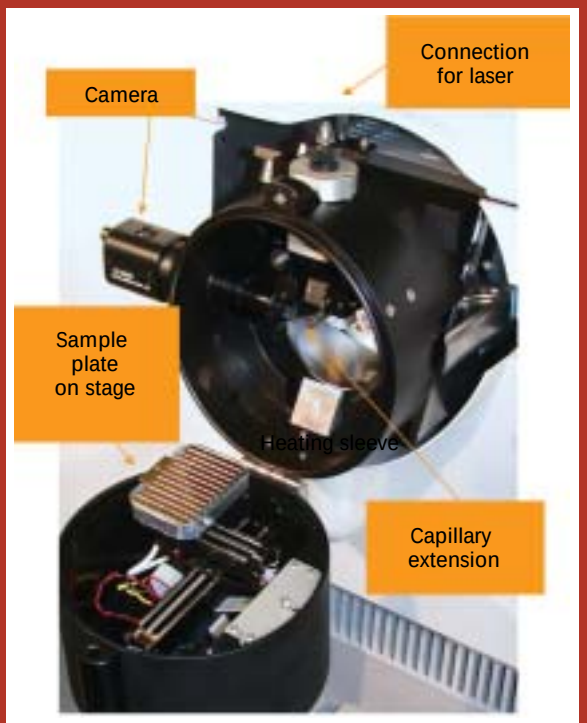


Figure 2. Ion trap MS fitted with an AP-MALDI source. The source chamber, which remains at atmospheric pressure, is shown in its open position to expose the inner workings. The sample plate, with positions for 96 samples, is mounted on a movable stage on the left half of the source. When the chamber is closed, the stage moves the plate so that each sample location can be positioned in front of the capillary extension tube, which is at the entrance to the ion optics and vacuum system of the ion trap. The laser is connected to the top of the source using a fiber optic cable. Ions produced by the vaporization event are drawn into the ion trap by a combination of vacuum and electric fields. The camera, in conjunction with the data system PC display, is used to assure correct positioning of each sample location and to verify laser operation. (Image courtesy of Agilent Technologies, Palo Alto, California, USA.)

easily requires 18 or more hours to carry out a comprehensive analysis. Clearly, 2-D LC–ESI MS alone will not meet the demand for the higher analytical throughput that is increasingly required by ongoing expansion in proteomic research.

AP-MALDI Ion Trap MS

Decoupling the ionization and detection functions in a MALDI-TOF mass spectrometer makes it possible to relocate the MALDI source outside the analyzer's vacuum enclosure and operate it at atmospheric pressure. Atmospheric pressure (AP)-MALDI, as this innovation is called, provides a soft ionization that is particularly well suited to the MS analysis of biomolecules and polymers, as well as small molecules. Potential replacements for the linear TOF analyzer that integrates well with the MALDI source include both quadrupole-TOF hybrids and the ion trap MS used in LC–ESI MS instruments (5). Integrating a MALDI source with an ion trap mass spectrometer completely isolates ion generation from mass detection. Any residual effects imparted by the ionization process are removed by the "store-and-analyze" operation of the trap. As a result, variations in source parameters have no effect on either the accuracy or the resolution of the mass spectrometer. Figure 1 is a schematic of an AP-MALDI source and Figure 2 shows an actual AP-MALDI ion trap MS instrument, indicating the principal components.

Among mass spectrometers, the ion trap is the instrument of choice for performing MS-MS experiments in which critical peptide sequence information is sought. The ion trap's unique capability to store, concentrate and select both precursor and fragmentation product ions through several stages of MS ideally suits it for proteomics applications. Some ion trap instruments incorporate additional ion selection and exclusion routines that make it possible to improve the breadth and quality of both the MS and MS-MS spectra produced. These improvements in data acquisition enable a greater amount of useful information to be extracted from an experiment than might otherwise be the case. Examples of such routines include automated ramping of the voltage during fragmentation in order to optimize the creation of product ions, including diagnostically important b- and y-series ions, and "on-the-fly" exclusion of both redundant mass information and noise, which can negatively impact both resolution and sensitivity. Taken together, these features enhance the overall information content

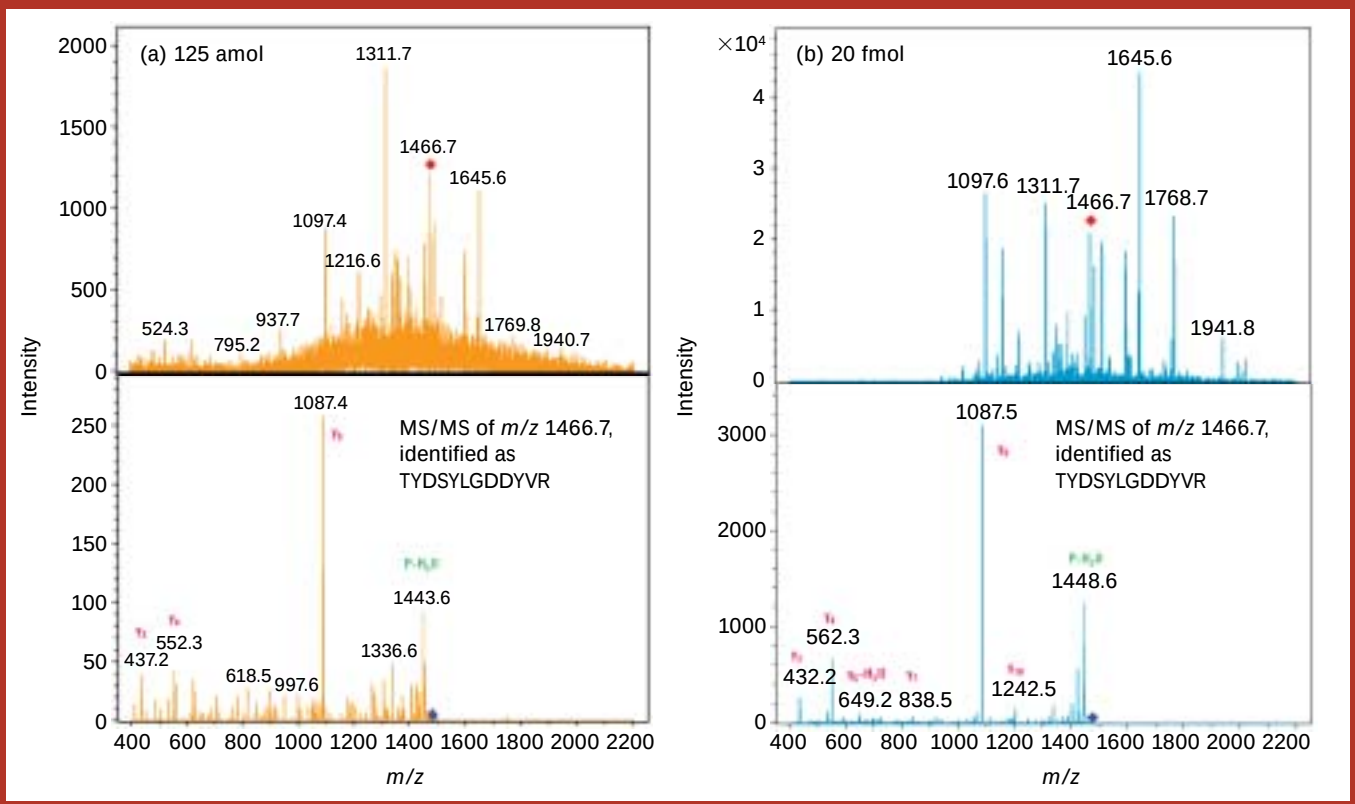


Figure 3. High-sensitivity MS-MS analysis. Shown here are the MS and MS-MS spectra of a commercially available tryptic digest of bovine apotransferrin obtained by analyzing 125 amol (left) and 20 fmol (right). The resemblance is obvious despite the 160-fold difference in amount. The product ion spectra of *m/z* = 1466.7, selected automatically during MS-MS operation, are shown. Several diagnostic b- and y-series ions can be identified.

of the data and shorten analysis times. Table I outlines and compares the respective attributes of the AP-MALDI ion trap MS and a conventional MALDI-TOF MS.

While the integration of an AP-MALDI source with an ion trap mass spectrometer was seen as an important development, the original implementation had a protein digest detection limit of about 20 fmol — insufficient for a significant number of proteomics analytical applications. Refinements to AP-MALDI sources and ion trap mass spectrometers have remedied this problem and increased sensitivity 100-fold, enabling the AP-MALDI ion trap to routinely demonstrate a mid-attomole range detection limit for mixtures of tryptic digests of protein standards (Figure 3). This dramatic improvement in sensitivity was accomplished primarily by raising the temperature of the countercurrent gas stream used to "cool" the plume of laser-ejected ions as they transit the interval from the MALDI stage to the ion trap. Experimental results indicate that the sensitivity increase is associated with the declustering of matrix ions by the warm countercurrent gas stream. Once declustering occurs, the matrix ions are barred from entering the analyzer in significant numbers by tuning the ion optics of the instrument to reject ions below mass 300. Currently, lines of research are being directed to achieve additional increases in sensitivity by optimizing ion transport into the ion trap (6).

Because a proteomics sample can contain several proteins of varying amounts, it is essential that the mass spectrometer scans quickly for enough information to identify all components of the mixture before the sample is exhausted. A scan speed of 26,000 amu per second of this system fulfills this requirement. An experiment devised to test the ability of the AP-MALDI ion trap to identify protein components in mixtures utilized a model system composed of 1 fmol each of apotransferrin, BSA and catalase. The standard digests were combined, and an aliquot that included 1 fmol of each digest was spotted onto a target plate. Analysis of the MS-MS product ion spectra identified the three proteins as the only significant matches (5). In another experiment, the AP-MALDI ion trap was estimated to have a dynamic range of 20, as determined by its ability to identify apotransferrin from its digest peptides (5 fmol) in the presence of a signifi-

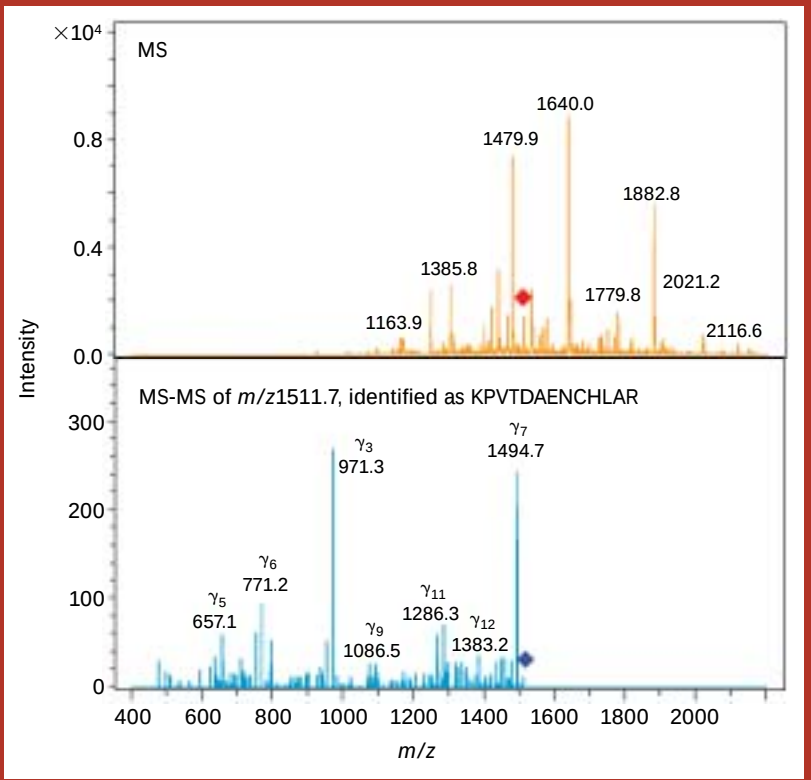


Figure 4. AP-MALDI ion trap dynamic range. A mixture of the tryptic digests of BSA and apotransferrin (100 and 5 fmol, respectively) was prepared. An MS-only spectrum (top) was used for automatic precursor selection. While peaks from the tryptic digest of BSA dominates this spectrum, the MS-MS spectra from the precursor at *m/z* 1511.6 indicate the presence of carboxymethylated Cys, which conclusively identifies apotransferrin and verifies a dynamic range of at least 20 for analysis of apotransferrin in the presence of higher concentrations of BSA.

cantly higher amount (100 fmol) of a BSA protein digest (Figure 4). The precise dynamic range of the instrument will vary from one experiment to the next, depending upon factors such as instrument settings, the structure of the proteins digested, the protease used and the digest conditions — all of which can significantly influence the instrument's response. It is anticipated that optimization along these lines will yield additional improvements in dynamic range (7, 8).

Comparing ESI MS and AP-MALDI MS Applications

Because MALDI MS (or AP-MALDI MS) and ESI MS have distinctive strengths and areas of application in proteomics analysis, a well-equipped proteomics laboratory probably would use both types of instrumentation. This normally would require purchasing and maintaining two dedicated mass spectrometers. The development of the AP-MALDI source makes it possible to interchange a MALDI and ESI source on a single ion trap mass spectrometer. Because both sources operate at atmospheric pressure, there is no need for vacuum pump-down or venting; source changeover can be accomplished in just a few minutes. In general, AP-MALDI is more suitable for the rapid analysis of relatively simple protein digest mixtures. Normally, it takes 1–2 hours to set up and run a dozen AP-MALDI samples. By contrast, a single LC–ESI MS-MS analysis, on average, takes 1–2 hours. Considering that a single 2-D gel separation can produce some 800 protein spots, prudence is required to decide how best to allocate these resources. Table II highlights and compares the respective capabilities for protein digest analysis of the AP-MALDI ion trap MS and the LC–ESI ion trap MS systems.

The MALDI process generally delivers singly-charged ions to the analyzer. By contrast, ESI produces multiply-charged ions under the conditions normally used to analyze protein digests. The charge state of an ion influences the fragmentation process under MS-MS conditions, and different peptide sequence information often is obtained from the two ionization techniques, even if the same peptides are examined. Given this fact, it is likely that a combination of data acquired on the same sample by both techniques will improve sequence coverage and, therefore, potentially increase both the efficiency of subsequent database matching and the confidence level of protein identity assignments. Figures 5 and 6 demonstrate the value of this approach in the analysis of the protein digests of apotransferrin and BSA using the ion trap with both the ESI and AP-MALDI sources. In each case, match scores and sequence coverage are greater for the combined data than those produced by either technique alone, leading to higher confidence in the findings. Table III lists the conditions for this experiment.

Table II. Comparison of AP-MALDI and LC–ESI Capabilities

AP-MALDI Ion Trap	LC–ESI Ion Trap
Rapid analysis of protein digests	Comprehensive analysis, including minor sample components and poor MALDI responders
Mid-range attomole sensitivity	Mid-range attomole sensitivity
MS-MS fragmentation for successful database search	Rich fragmentation of peptides (+2 ions)

Table III. Experimental Conditions for the Digest Peptide MS-MS Analysis of Bovine Serum Albumin and Apotransferrin by AP-MALDI and ESI MS

	ESI MS	AP-MALDI MS
SAMPLE		
	1 pmol/µL BSA or apotransferrin digest (Michrom BioResources, Auburn, CA, USA) in 15% acetonitrile/85% water with 0.1% formic acid; inject 0.5 µL (500 fmol on column)	10 fmol/µL BSA digest and 1.5 µg/µL α cyano-hydroxycinnamic acid (CHCA) in 25% methanol/19% isopropyl alcohol/56% water with 0.75% acetic acid; spot 0.5 µL on plate and allow to dry; these concentrations resulted in the same sequence coverage (approximately 30%) for both techniques
LC		
Column	Reverse-phase 300 Å, C18, 0.15 x 150 mm, 5 µ	
Mobile phase	Flow rate: 1 µL/min; (A) water with 0.1% formic acid, (B) acetonitrile with 0.1% formic acid	
Gradient	2% B for 5 min, then 40% at 40 min, 60% at 42 min, 60% at 45 min, 2% at 46 min	
Stop time	60 min; post time: 15 min	
Injection program	valve to bypass at 5 min	
MS		
Ionization mode	positive ion microspray	positive ion
Vcap	-4000 V	-3000 V
Nebulizer	10 psig	
Drying gas	10 L/min	5 L/min
Drying gas temp ºC	150 ºC	300 ºC
Skimmer	35 V	40 V
Cap exit voltage	100 V	260 V
Scan range	300–1800	400–2200
Precursors	2	30
ICC	20,000 or 200 ms	200,000 or 535 ms
Averages	MS = 4; MS 2 = 5	MS = 24; MS 2 = 16
Isolation width	4 amu	4 amu
Frag. amplitude	1.2 V	1.5 V
SmartFrag	30–200%	30–200%
Active exclusion	2 spectra, 0.5 min; exclude singly-charged ions, prefer doubly-charged ions	1 spectrum, 20 min
Total acq. time	60 min	5.1 min
Spectrum Mill search conditions		
Search	Batch-Tag (MS-MS)	
Database	NCBI-mammalian	
Enzyme	trypsin	
Cys	carboxymethyl	
Max missed cleavages	1	
Instrument type	ESI ion trap or MALDI ion trap	

rate quality data from noise, batch processing of data files, automated database searching, *de novo* sequencing and the selective processing and display of data to reveal hidden patterns and trends. All of these routines provide powerful new ways to reduce the proteomic MS data processing load and analysis time while improving the quality of results and the output of useful information.

Figure 6 is a typical example of an MS data summary created with the Spectrum Mill MS proteomics workbench (Agilent Technologies, Palo Alto, California, USA), an informatics tool kit consisting of integrated data management routines designed specifically for MS proteomic applications. In another example that illustrates the utility of this type of software (not shown), search results from 20,000 spectra obtained from

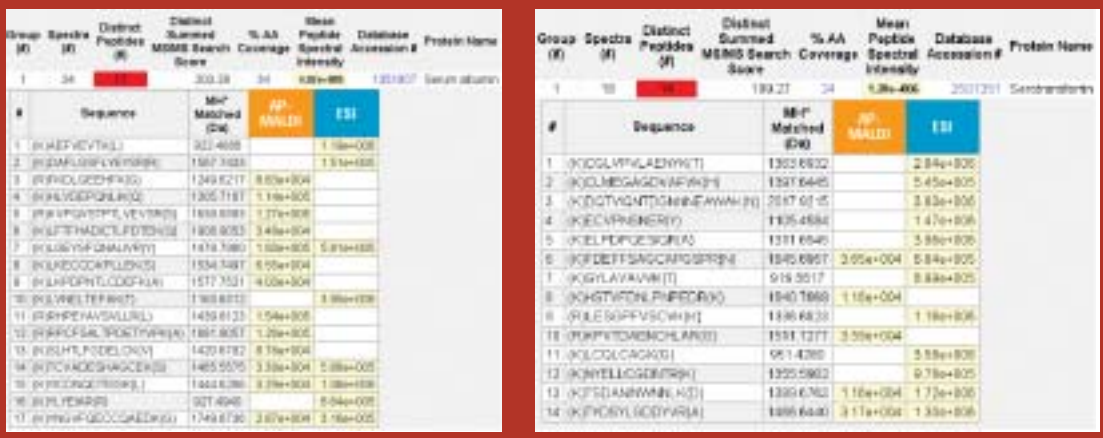


Figure 6. Comparison of AP-MALDI and ESI data files. Combining data from both techniques improves sequence coverage in protein digest analysis because each type of ionization produces some unique peptide identifications. In the examples shown here, ESI provides superior sequence coverage in the analysis of apotransferrin than AP-MALDI, while the reverse is true, but somewhat less dramatic, for BSA. (Image courtesy of Agilent Technologies, Palo Alto, California, USA.)

24 LC–MS-MS analyses (two samples, 12 fractions each) were extracted, combined and consolidated into a single table of protein identifications and abundances. A comparable review and summary performed manually would have taken days, compared to the few hours of computer processing time required by this application.

Conclusion

It is no longer sufficient to extract out and sequence an unknown target protein in isolation. Increasingly, there is a need to understand, in precise structural detail, the subcellular system encompassing a cycle of protein interactions. Researchers need to know which proteins are involved at what concentration and in which cell location. These questions and many more like them will be addressed by the development of increasingly powerful protein analysis tools and methodologies, such as those described here, as well as by new ways of integrating and leveraging the information these platforms generate.

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Jose E. Meza, Donghui Yi, Christine A. Miller and Patrick D. Perkins are senior applications chemists at Agilent Technologies. Patrick D. Perkins can be reached at 5301 Stevens Creek Boulevard, Santa Clara, California 95051 USA; e-mail pat_perkins@agilent.com.