Proteomic Profiling of a Snake Venom Using High Mass Detection MALDI-TOF Mass Spectrometry

Oscar Yanes^{*†} and Francesc X. Avilés

Institut de Biotecnologia i Biomedicina and Departament de Bioquímica i Biol. Mol., Universitat Autònoma de Barcelona, Bellaterra (Barcelona), Spain

Ryan Wenzel,[†] Alexis Nazabal,[†] and Renato Zenobi

Department of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland

Juan J. Calvete

Instituto de Biomedicina de Valencia, C.S.I.C., Valencia, Spain

Proteomic profiling involves identification and quantification of protein components in complex biological systems. Most of the mass profiling studies performed with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) have been restricted to peptides and small proteins (<20 kDa) because the sensitivity of the standard ion detectors decreases with increasing ion mass. Here we perform a protein profiling study of the snake venom *Sistrurus miliarius barbouri*, comparing 2D gel electrophoresis and reversed-phase high-performance liquid chromatography (HPLC) with a high mass cryodetector MALDI-TOF instrument (Macromizer), whose detector displays a uniform sensitivity with mass. Our results show that such MS approach can render superior analysis of protein complexity compared with that obtained with the electrophoretic and chromatographic approaches. The summation of ion impacts allows relative quantification of different proteins, and the number of ion counts correlates with the peak areas in the reversed-phase HPLC. Furthermore, the sensitivity reached with the high mass cryodetection MS technology clearly exceeds the detection limit of standard high-sensitivity staining methods. (J Am Soc Mass Spectrom 2007, 18, 600–606) © 2007 American Society for Mass Spectrometry

Protein profiling has become a powerful method for analyzing changes in global protein expression patterns in biological systems as a function of developmental, physiological, and disease processes. Two-dimensional (2D) gel electrophoresis has been established for many years as the primary tool for detecting proteins present in an organism or a complex biological extract. However, 2D gel electrophoresis has some limitations: limited solubility of hydrophobic and membrane proteins, limited dynamic range, difficulty in focusing highly basic and acidic proteins, poor sensitivity, poor quantitation, and finally, the method is not amenable to automation. The limitations associated with gel electrophoretic analysis of peptides

and small proteins (<20 kD) have stimulated interest in mass spectrometry as an alternative strategy. In this sense, MALDI-TOF mass spectrometry has partially replaced or complemented gel-based approaches for studying the proteomic profile of the low mass components of many complex biological samples such as serum [1] or tissues [2]. Imaging MS [2] and chip-based technologies such as surface-enhanced laser desorption/ionization (SELDI-MS) [3] are relatively new approaches that take advantage of the established features of MALDI MS (mass accuracy, sensitivity, reliability, and high-throughput) to detect and identify peptides and proteins. Also, 1D and 2D liquid chromatography coupled to detection systems such as UV and/or MALDI, have been gradually introduced for the analysis and profiling of intact (top down proteomics) [4, 5] and digested proteins (bottom up proteomics) [6] in complex biological mixtures. MALDI is a soft ionization method and produces predominantly singly charged molecular ions regardless of the molecular weight. Hence, the spectra are relatively easy to interpret.

Published online January 3, 2007

Address reprint requests to Dr. F. X. Avilés, Institut de Biotecnologia i Biomedicina and Departament de Bioquímica i Biol. Mol., Universitat Autònoma de Barcelona, 08193 Bellaterra (Barcelona), Spain. E-mail: Francesc.Xavier.Aviles@uab.es

^{*} Also at the Department of Chemistry and Applied Biosciences, Swiss Federal Institute of Technology (ETH), Zürich, Switzerland.

[†] CovalX GmbH, Schumacherweg 15, CH-8046, Zürich, Switzerland.

Up to now, most of the profiling studies carried out using MALDI-TOF mass spectrometry have been performed using instruments with standard ion detectors, such as microchannel plates (MCP), which work by ion-to-electron conversion following the impact of high velocity ions on the detector surface, initiating an electron cascade. However, the conversion efficiency is extremely low for large, low velocity ions, and an exponential decrease in efficiency can be readily observed at higher masses [7,8]. Additionally, MCP channels have a very slow refresh time, about several milliseconds. This means that when an ion impacts a channel, it is turned off for the duration of the analysis (microseconds). When measuring complex samples that contain ions over a broad mass range, the lower intensity ions will saturate some channels of the detector, preventing the analysis of the higher ions, again leading to a decrease in sensitivity for larger mass ions [9]. As an alternative, cryodetection is an ion detection method that does not suffer from a decrease in sensitivity for high m/z ions or from a decrease in sensitivity from complex samples [10,11]. The capacity of the cryodetector technology [12], or recently proposed equivalent substitutes [13] for intact protein analysis of highly complex protein mixtures, with uniform sensitivity, independent of mass, provides a new opportunity to establish MALDI mass spectrometry in the field of protein profiling for all mass ranges.

In the present work, we demonstrate the protein profiling of crude snake venom from *Sistrurus miliarius barbouri* using cryodetection MALDI TOF mass spectrometry. The results were compared with those obtained by Juárez et al. [14] using 2D gel electrophoresis and reversed-phase HPLC liquid chromatography (LC), highlighting the sensitivity of cryodetection and its ability for relative quantification of different analytes in the same spectrum. Overall, we demonstrate the power of this new technology to be implemented as a mass spectrometry-based quantitative proteomic profiling strategy.

Experimental

Cryodetection MALDI-TOF MS

A linear MALDI-TOF mass spectrometer equipped with a cryogenic superconducting tunnel junction array detector (VTT Technical Research Center of Finland, Espoo, Finland) was used for this study (Macromizer, Comet AG, Flamatt, Switzerland) [the Macromizer instrument is no longer commercially available.] The Macromizer instrument is designed to optimize ion transmission onto the small area of the cryodetector array. Every part of the instrument, including all necessary ion optics, laser, and electronics, were designed to enhance detection of high mass ions. For ionization, a 337 nm nitrogen laser (VSL 337ND-S, Spectra-Physics, Mountain View, CA), capable of producing energies up to 90 mJ with 4 ns pulse widths at 5 Hz repetition rate is focused to a spot size of ~ 0.1 mm. A detailed

description of the Macromizer design is given by Wenzel et al. [12]. For the work described in this paper, 15 kV sample plate voltage, 3 kV extraction voltage, 8 kV Einzel lens voltage, and 4 μ s delayed extraction time were used. Spectra were acquired by averaging 200 to 250 shots and processed using the Data Analyzer software (Comet).

MCP MALDI-TOF MS

MALDI mass spectra using a microchannel plate (MCP) detector were obtained using an Ultraflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a 337-nm nitrogen laser, a gridless ion source, delayed-extraction (DE), and a 2-GHz digitizer. The instrument was operated in linear mode by applying an acceleration voltage of 20 kV. The delay time used was 500 ns. Mass spectra were acquired by averaging 300 to 400 shots.

Sample Preparation

The matrix 2,6-dihydroxyacetophenone (Fluka Chemie, Buchs SG, Switzerland) was dissolved at a concentration of 10 mg/mL in 30% ACN/20 mM ammonium citrate in water. Lyophilized venom from *Sistrurus miliarius barbouri* was purchased from Latoxan Serpentarium (Rosans, France). Lyophilized venom was dissolved in water and insoluble material was removed by centrifugation at 13,000 g for 10 min at room temperature. The sample was diluted to a desired concentration with water, mixed at a 1:1 vol/vol ratio with matrix solution and 0.5 μ L of this solution was applied onto the MALDI target plate with the dried-droplet method [15].

2D SDS PAGE

Protein composition of lyophilized venom from *Sistrurus miliarius barbouri* was analyzed by 2D-SDS-PAGE using an IPGphor instrument (Amersham Biosciences, Uppsala, Sweden). For the first dimension (IEF), 500 to 1000 μ g of total venom proteins (in 250 μ L of 8 M urea, 4% CHAPS, and 0.5% IPG buffer) were loaded on a 13 cm IPG strip (pH range 3 to 10) using the following focusing conditions: 30 V for 6 h, 60 V for 6 h, 500 V for 1 h, 1000 V for 1 h, and 8000 V for 2 h. Electrophoretic separation (second dimension) was done in a 16 cm 15% acrylamide gel. Coomassie brilliant blue (Amersham Biosciences, Uppsala, Sweden) was employed for protein staining.

Reverse Phase HPLC

For reverse-phase HPLC separations, 2 to 5 mg of crude venom were dissolved in 100 μ L of 0.05% trifluoroacetic acid (TFA) and 5% acetonitrile, and insoluble material was removed by centrifugation at 13,000 g for 10 min at room temperature. Proteins in the soluble material were separated using an ETTAN LC HPLC system (Amer-

sham Biosciences, Uppsala, Sweden) and a Lichrosphere RP100 C18 column (250 × 4 mm, 5 μ m particle size) eluted at 1 mL/min with a linear gradient of 0.1% TFA in water (Solution A) and acetonitrile (Solution B) (isocratically (5% B) for 5 min, followed by 5 to 15% for 20 min, 15 to 45% B for 120 min, and 4 to 70% B for 20 min). Protein detection was at 215 nm.

Results and Discussion

Quantitative Profiling of the Snake Venom

The increased sensitivity for detection of high mass ions using the cryodetection MALDI-TOF mass spectrometer is shown in Figure 1a. The spectrum is displayed divided into 5 to 35 kDa and 35 to 110 kDa ranges for easier visualization, although it was acquired in a single acquisition. It shows a mass-independent signal response, further validated when compared with the HPLC peak areas (see later on and Figure 2a, b, c and Table 2). In contrast, when analyzing the same sample with a conventional MCP detector the spectrum clearly displays a dramatic signal decrease with increasing mass. Furthermore, fewer ion signals are observed with the MCP detector, thus not representing the same

complexity of the biological sample observed by cryodetection MS. Figure 1b shows one of the best mass spectra that we could achieve to get a mass profiling including high mass peaks with the MCP detector from our Ultraflex MALDI-TOF instrument (see the Experimental section). All parameters were optimized and replicates were done on different days.

The crude venom of *Sistrurus miliarius barbouri* was fractionated by reverse-phase HPLC (Figure 2a). The characterization of the proteins present in each fraction was previously done by Juárez et al. [14], using SDS-PAGE, MALDI-TOF MS, Edman degradation (N-terminal sequencing), and tandem mass spectrometry in a quadrupole-linear ion trap instrument (data not shown). Isolated proteins could be unambiguously assigned to protein families present in the Swiss-Prot/TrEMBL database [14]. Some representative examples are included in Table 1. Separation of the protein components of the crude venom extract by 2D gel revealed the presence of four or five major protein spots (Figure 2b), and the presence of isoforms for the serine protease (protein 2) [14].

An additional feature of cryodetector MS is its capability to measure total kinetic energy, not just the arrival

Figure 1. High-mass cryodetection (a) and microchannel plate (b) MALDI-TOF mass spectra of the *S. miliarius barbouri* venom extract. 0.8 μ g of total protein was deposited onto the MALDI plate without previous fractionation. Numbers in brackets indicate proteins shown in Table 1 and previously identified [14].

Figure 2. Separation of *S. miliarius barbouri* venom proteins by reverse-phase HPLC (a) and two-dimensional gel electrophoresis (b). Total venom proteins (1000 μ g) were subjected to IEF using 13 cm IPG strip followed by SDS in a 15% acrylamide (16 cm). Scatterplot of individual ion events m/z versus the normalized kinetic energy of each impact (c). Numbers in brackets indicate proteins shown in Table 1 and previously identified [14].

time of the different ions. Energy is determined for each ion impact and is displayed as normalized kinetic energy versus m/z ratio. Figure 2c shows the energy versus m/z scatterplot of data represented in Figure 1a. The total number of ion counts in the 5 to 120 kDa mass range was 64,459. Ion counts for individual proteins of the extract and their respective percentage and relative values were also calculated (see Table 2). A key finding is the excellent correlation between the chromato-

graphic relative peak areas (Figure 2a), if followed at 214 nm, and the relative ion counts of each protein within the cryodetector MS (see Table 2). Ultraviolet absorbance at 214 nm is widely recognized as a quantitative tool to determine the relative abundance of proteic components in a sample, with an accuracy of the same or superior level as gel electrophoresis with chromogenic staining. Although the latter, in the 2D mode, also correlates qualitatively, accurate quantitation of the

Table 1. Assignment of the reversed-phase isolated fractions of *Sistrurus barbouri* venom to protein families by N-terminal Edman sequencing and MALDI-TOF mass spectrometry ([14])

HPLC fraction	N-terminal sequencing	Isotope-averaged MALDI-TOF mass ($\pm 0.2\%$)	Protein family
1	HLIQFETLIMKIAGRSGVFW	13980	Phospholipase A2
2	VIGGNECNINEHRSL	27 kDa ^a	Serine proteases
3	NPEHQRYVELFIVVD	23 kDa ^a	PI-metalloprotease
4	Blocked	48.5 kDa ^a	PIII-metalloprotease

^a Apparent molecular mass determined by SDS-PAGE after sample reduction with β -mercaptoethanol.

Table 2. Relative peak areas and ion counts values are the ratio of each protein value to the highest one (i.e., protein 4)

Protein	HPLC			Cryodetection MALDI-TOF MS		
	Area ^a	Peak area (%)	Relative peak area	Ion counts ^b	Ion counts (%)	Relative ion counts
1	6.019,618	17.04	0.76	2181	3.38	0.63
2	7.046,113	19.95	0.89	3276	5.08	0.95
3	3.911,487	11.07	0.49	1566	2.42	0.45
4	7.855,834	22.23	1	3447	5.34	1

^aTotal area (mAU/ml): 50.394,5.^bTotal ion counts: 64.459.

spots is cumbersome and very dependent of the capability of the protein species to bind the chromogenic dye used for staining [16]. As examples, the protein 4 of 48.5 kDa (PIII metalloprotease) seems to be the most abundant protein in our analysis, both in the mass spectrum and RP-HPLC (Figures 1a and 2a). This protein represents 5.3% of the total number of ions detected by cryodetector MALDI-TOF. Relative peak areas for proteins 1 and 3, PLA₂ (phospholipase A₂) and PI-metalloproteinase from the RP-HPLC also fit quite well with the relative ion counts derived from the cryodetector MS data (see Table 2). For protein 2 (serine protease), different isoforms appear in adjacent chromatographic peaks as previously described [14], and the peak areas have been integrated as one value that also fits very well with the ion counts of the cryodetector MS (see Table 2).

One of the main disadvantage of cryodetection MALDI-TOF is its relatively low-resolution for the present mass spectrometric instrument. This is mainly due to the STJ, which is an inherently slow detector [12], together with the fact that Macromizer is a linear TOF instrument (equipped with a 1.5 m drift region), and the delay pulse extraction over a wide mass range is generally problematic or impossible. These issues, for the time being, may prevent the detection of isoenzymes with very similar molecular mass until such technical constraints are solved. This is the case of the different isoforms of a serine-protease (protein 2 in this work) previously identified by Juárez et al. [14]. Whereas the 2D-gel and the RP-HPLC show spots with different pI and the very similar MW and adjacent peaks in the RP-HPLC chromatogram, respectively (see Figure 2a, b), the heterogeneity of isoforms (peak at ~27.4 kDa) cannot be resolved as individual species from the cryodetection MALDI-TOF mass spectrum of Figure 1a and the energy versus m/z scatter plot of Figure 2c. On the other hand, the high mass ion complexity (50 to 120 kDa) observed with the cryodetection MALDI-TOF could neither be detected with RP-HPLC nor with the 2D gel electrophoresis. In the case of the RP-HPLC, it would be explained because the characteristic reversible adsorption/desorption of analytes (by adjusting the polarity of the mobile phase) on the reversed-phase chromatography becomes sometimes irreversible with high mass proteins (>50 kDa)

due to their high hydrophobicity (specially using C18 and C8 ligands).

Sensitivity of High-Mass Cryodetection MALDI-TOF MS

High sensitivity is a crucial aspect of proteomics research. To date, the available stains for visualizing proteins in a 2D gel yield relatively poor sensitivity or poor peptide recovery for mass spectrometric investigation of in-gel digests. In addition, the limited dynamic range of these stains has made it difficult to rigorously and reliably determine differences in protein quantities (especially for silver staining). Figure 3 shows the energy versus m/z scatter plot of the snake venom extract at decreasing amounts of total protein. While the detection limit for a single protein using (MS compatible) silver staining lies at around 40 ng [17], cryodetection MS seems to be at least 10 times more sensitive. Previous cryodetector research showed sensitivities as low as 1 femtomole for a single protein sample of an antibody [18]. Limits of detection were defined as the lowest concentration points for which signals were still reproducibly detected with a signal-to-noise ratio ≥ 2 . Figure 3 shows how the total number of ion counts is decreasing to the amount of total protein deposited onto the MALDI plaque, but the ion count percentage of single proteins is maintained independently of their mass (see Table 3). Only a few proteins above 60 kDa could not be detected at very low concentrations of total protein. The detection limit for a feasible relative quantification of different proteins in this sample with the Macromizer instrument was established as 10 ng of total protein. It is worth mentioning that there is under advanced development a new generation of related high mass MALDI-TOF detector, the ICD, which does not require the cryostat and shows a very similar sensitivity (and signal uniformity) along a broad mass range with protein samples similar to the ones here analyzed [13].

Conclusions

In the present study, two completely different MALDI-TOF mass spectrometers were used to com-

Figure 3. Scatter plot of individual ion events in the high-mass cryodetector, m/z versus the kinetic energy of each impact; 100, 30, 20, and 10 ng of total venom proteins were deposited onto the MALDI plaque.

pare their performance exploring the mass profiling of a snake venom extract, fitted with two different detectors, either the currently used MCP or a high-mass cryodetector. Although the main difference concerns their detectors, other m/z biases in the instruments (e.g., different ion transmissions) cannot be disregarded.

The snake venom extract is not an extremely complex biological sample, so we have taken advantage of the mass-independent protein detection and very high sensitivity of cryodetection MS to obtain one of the most complete mass profiling to date of this biological (crude) extract without any previous fractionation step. Obviously, we cannot assert that it is the true sample

Table 3. Ion count percentage of single proteins at decreasing amounts of total protein

Total protein (ngr.)	Total counts	Percentage of individual proteins from total protein by ion signal at specific mass ranges (kDa)				
		7–8	11.5–12.2	14–15	23–24	49–51
100	9895	12.79	1.26	6.08	5.50	6.44
30	6355	13.13	1.74	7.94	6.89	10.63
20	6638	10.03	1.79	8.24	6.83	10.36
10	2475	22.26	2.02	10.10	7.11	8.48
Average		14.55	1.70	8.09	6.58	8.98
Std. dev.		5.32	0.31	1.64	0.72	1.94
Coef. variation		36.55	18.65	20.31	11.07	21.62

complexity as there may be protein species present in the sample that are at levels too low to be visualized. However, if this is the case, neither 2D-gel electrophoresis nor HPLC data seem to detect them.

Theoretical and practical considerations [12], and assuming absence of bias in ion transmission, lead to the conclusion that STJ ion counts, displayed as normalized kinetic energy versus m/z ratio, represents the closest true ion yield of the MALDI process. This shows the potential of cryodetection MALDI-TOF MS (or equivalent next generation high mass detectors) as a promising tool for mass spectrometry-based quantitative proteomic profiling strategies. Considering that in such instruments signal suppression effects due to the detector are absent, quantification of the protein content using MALDI mass spectrometry will only depend on the relative ionization efficiencies of the different compounds within a complex mixture, again assuming absence of bias in ion transmission. For this reason, complementary previous fractionation and separation procedures should be performed in future proteomic strategies to increase the ionization efficiency of as many proteins as possible.

Although over the last several years there have been many advances for mass spectrometry in ionization methods, mass separation, and fundamental understanding, one of the most important steps in this process, the detection of ions, has remained virtually unchanged. The present study demonstrates that novel detection technologies, exemplified by high-mass cryodetection, may open up new strategies and applications for mass spectrometry in high-throughput proteomic projects.

Acknowledgments

This work was supported by grant no 200020-103616 (Swiss National Science Foundation), grants GEN2003-20642-C09-05, and BIO2004-05879 (Ministerio de Ciencia y Tecnología, MCYT, Spain), and by Centre de Referència en Biotecnologia (Generalitat de Catalunya, Spain). OY acknowledges a fellowship from MCYT.

References

- Villanueva, J.; Philip, J.; Entenberg, D.; Chaparro, C. A.; Tanwar, M. K.; Holland, E. C.; Tempst, P. Serum Peptide Profiling by Magnetic Particle-Assisted, Automated Sample Processing and MALDI-TOF Mass Spectrometry. *Anal. Chem.* **2004**, *76*, 1560–1570.
- Caldwell, R. L.; Caprioli, R. M. Tissue Profiling by Mass Spectrometry: A Review of Methodology and Applications. *Mol. Cell. Proteom.* **2005**, *4*, 394–401.
- Issaq, H. J.; Veenstra, T. D.; Conrads, T. P.; Felschow, D. The SELDI-TOF MS Approach to Proteomics: Protein Profiling and Biomarker Identification. *Biochem. Biophys. Res. Commun.* **2002**, *292*, 587–592.
- Kelleher, N. L. Top Down Proteomics. *Anal. Chem.* **2004**, *76*, 197A–203A.
- Sheng, S.; Chen, D.; Van Eyk, J. E. Multidimensional Liquid Chromatography Separation of Intact Proteins by Chromatographic Focusing and Reversed Phased of the Human Serum Proteome: Optimization and Protein Database. *Mol. Cell. Proteom.* **2006**, *5*, 26–34.
- Zhang, N.; Li, N.; Li, L. Liquid Chromatography MALDI MS/MS for Membrane Proteome Analysis. *J. Proteome Res.* **2004**, *3*, 719–727.
- Twerenbold, D.; Gerber, D.; Gritti, D.; Gonin, Y.; Netuschil, A.; Rossel, F.; Schenker, D.; Vuilleumier, J. L. Single Molecule Detector for Mass Spectrometry with Mass Independent Detection Efficiency. *Proteomics* **2001**, *1*, 66–69.
- Brunelle, A.; Chaurand, P.; DellaNegra, S.; LeBeyec, Y.; Parilis, E. Secondary Electron Emission Yields from a CsI Surface Under Impacts of Large Molecules at Low Velocities [5x10(3)–7x10(4) ms⁻¹]. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 353–362.
- Chen, X. Y.; Westphall, M. S.; Smith, L. M. Mass Spectrometric Analysis of DNA Mixtures: Instrumental Effects Responsible for Decreased Sensitivity with Increasing Mass. *Anal. Chem.* **2003**, *75*, 5944–5952.
- Frank, M.; Mears, C. A.; Labov, S. E.; Benner, W. H.; Horn, D.; Jaklevic, J. M.; Barfknecht, A. T. High-Efficiency Detection of 66,000 Da Protein Molecules Using a Cryogenic Detector in a Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometer. *Rapid Commun. Mass Spectrom.* **1996**, *10*, 1946–1950.
- Twerenbold, D. Cryogenic Particle Detectors. *Rep. Prog. Phys.* **1996**, *59*, 349–426.
- Wenzel, R. J.; Matter, U.; Schultheis, L.; Zenobi, R. Analysis of Megadalton Ions Using Cryodetection MALDI Time-of-Flight Mass Spectrometry. *Anal. Chem.* **2005**, *77*, 4329–4337.
- Wenzel, R. J.; Nazabal, A.; Zenobi, R. Comparison of Sensitivity and Saturation of MALDI-TOF Detectors for High-Mass Ions. *Proceedings of the 54th ASMS Conference on Mass Spectrometry*; Seattle, May 2006.
- Juarez, P.; Sanz, L.; Calvete, J. J. Snake Venomics: Characterization of Protein Families in *Sistrurus barbouri* Venom by Cysteine Mapping, N-terminal Sequencing, and Tandem Mass Spectrometry Analysis. *Proteomics* **2004**, *4*, 327–338.
- Hillenkamp, F.; Karas, M. Laser Desorption Ionization of Proteins with Molecular Masses Exceeding 10,000 Daltons. *Anal. Chem.* **1988**, *60*, 2299–2301.
- Rabilloud, T. Two-Dimensional Gel Electrophoresis in Proteomics: Old, Old Fashioned, but It Still Climbs Up the Mountains. *Proteomics* **2002**, *2*, 3–10.
- Rabilloud, T. Detecting Proteins Separated by 2D Gel Electrophoresis. *Anal. Chem.* **2000**, *1*, 48A–55A.
- Chaurand, P.; Hayn, G.; Matter, U.; Caprioli, R. M. *Proceedings of the 52nd ASMS Conference on Mass Spectrometry and Allied Topics*; May 2004, Nashville, TN.