Mass Spectral Analysis of Biological Agents Using the BioTOF Mass Spectrometer

Miquel D. Antoine, Micah A. Carlson, Willie R. Drummond, Otis W. Doss III, C. Scott Hayek, Anshu Saksena, and Jeffrey S. Lin

A miniaturized MALDI-TOF mass spectrometer has been in development at APL for the last several years. Besides meeting the mechanical design challenges in taking a bench-top–sized instrument and shrinking it down for easy and rapid deployment with autonomous operation, sample collection and preparation methods have been developed to analyze, detect, and identify biological compounds in an aerosol. Validation of sample treatment strategies was accomplished with two independent processing schemes by quantifying several metrics and determining the effects of sample processing protocols on those metrics.

INTRODUCTION

Matrix-Assisted Laser Desorption/Ionization (MALDI) Mass Spectrometry (MS) using a time-of-flight (TOF) mass analyzer is an established analytical technique routinely used to analyze a wide variety of biological compounds.[1–3] Small, low molecular weight substances (e.g., peptide fragments representative of proteineous material from a bacterial spore coat) to large, macromolecular complexes can be detected and identified with relative ease. However, ease of detection is often directly related to the sample preparation strategy employed.

During the MALDI mass spectrometric process, a given analyte is mixed with a matrix material, typically a small organic acid, on a probe surface and is allowed to dry. The sample is then inserted into the mass spectrometer where it is bombarded with photons from an ultraviolet laser. Desorbed ions are accelerated and directed into the field-free region of the mass analyzer and separated according to their mass-to-charge ratio (m/z). As the ions converge on the detector, their TOF, which is directly related to the mass of the ion, is recorded. A mass spectrum, i.e., a graphical plot of the data, is generated, with ion (peak) intensities recorded along the y axis and the m/z of detected ions on the x axis. The process is repeated several times with adjustments to the sample preparation protocol to ensure accurate and reproducible detection and identification of desorbed ions.

The BioTOF mass spectrometer is a miniaturized instrument developed to analyze biomolecular compounds.[4–8] Using MALDI, combined with a TOF mass analyzer, the BioTOF system is capable of analyzing a broad spectrum of compounds. To exploit this dynamic property for analyzing a large variety of molecules,

several experimental parameters must be considered to ensure optimal performance of the instrument.

SAMPLE PREPARATION

Aerosol Collection

The BioTOF mass spectrometer features an air-air virtual impaction collection system that outputs a concentrated aerosol into a five-jet real impactor and subsequently deposits the particles onto VHS cassette magnetic recording tape.[8] The tape is transferred to a module for deposition of the MALDI matrix onto the aerosolized sample spot. Sample treatment methods and incorporation of an internal standard are also conducted at this module. Following matrix application and drying, the sample is moved to the focal point of the laser, and upon laser firing, data are acquired from the detector. The data pass into a signal processing system that filters noise, converts the time signal to an *m*/*z*, determines a baseline, and identifies major peaks.

Enhancement in the performance of BioTOF is coincident with the development of novel technologies to increase the capture efficiencies of airborne particulates. Originally, aerosol particles were collected on dry, uncoated VHS tape, which resulted in low collection efficiencies of airborne particles. An uneven distribution of the collected sample was also evident. A distinct outer ring of material was clearly visible around a central spot, most likely due to particles bouncing off the tape surface and re-aerosolizing. With the laser focused to the center of the collected spot, a large portion of the sample was not being analyzed. Microbiological culturing studies of collected sample spots further indicated low levels of collected material. Subsequent analyses were conducted to examine the efficacy of coating the tape with "sticky" materials, such as mineral oil, to reduce the bounce and enhance collection at the center. During this investigation, it was determined that a very light coating of vacuum grease to the VHS tape surface prior to aerosol collection increased collection efficiency dramatically. MALDI mass spectra obtained from these samples displayed intense peaks with signal intensities greater than 10 orders of magnitude stronger than ions detected on uncoated tape. Unfortunately, this coating also had a major drawback; the tape began to slip during advancement and therefore generated alignment problems.

An alternative approach discovered during this process incorporated a water aerosol into the collection system between the air-air concentrator and tape impactor. Aerosolized water particles fed into the sample stream were found to increase the agglomeration of aerosol particles, thereby increasing their aerodynamic diameter. Consequently, when the hydrated particles collided onto the collection surface, particle bounce diminished considerably. A more defined center spot was clearly visible, and the distinct outer ring seen before was absent. Figure 1 shows fluorescent micrographs of polystyrene latex (PSL) beads aerosolized and collected on the surface of oil-coated tape (left) and uncoated tape (middle); the micrograph on the right shows PSL beads co-aerosolized with water on the surface of uncoated tape. The intensity of the fluorescence measured is directly related to the quantity of PSL beads captured on the tape surface. Automated counting software also enumerated collected particles. The benefits of an oil coating to the tape or injection of water with the PSL aerosol compared to the uncoated, dry tape are clearly evident by the lack of color in the center micrograph in Fig. 1.

MALDI mass spectral data of aerosolized *Bacillus globigii* (*Bg*) spores collected in a similar manner as the PSL beads are shown in Fig. 2. Comparison of ion signal intensities for the base peak (i.e., tallest) observed in three spectra—123.0, 7.2, and 38.0 mV, for coated tape, uncoated tape, and uncoated tape with co-aerosolized water, respectively—indicate that dry tape with water injection is a viable alternative to oil coating for improving collection and the corresponding MALDI MS analysis of aerosolized particles. Furthermore, since the water evaporates from the surface following collection, adverse effects on the performance of the tape drive system are prevented.

Exploration of additional solvents and methods to increase the collection of aerosolized particles is a continuing effort. Water has since been replaced with an organic solvent as the co-aerosolization reagent, and modified collection surfaces are envisioned in future design considerations for next-generation instruments.

Matrix Material

Another key component in the sample preparation process is the choice of MALDI matrix material, which, as noted earlier, is usually a small organic acid that absorbs the incident laser energy, thereby protecting the sample from thermal degradation. Upon desorption of

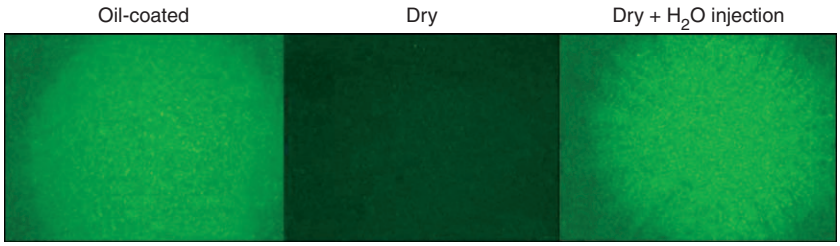


Figure 1. Fluorescent micrographs of polystyrene latex (PSL) beads aerosolized and captured on oil-coated VHS tape (left), uncoated tape (center), and uncoated tape with co-aerosolized water (right), illustrating particulate capture efficiency.

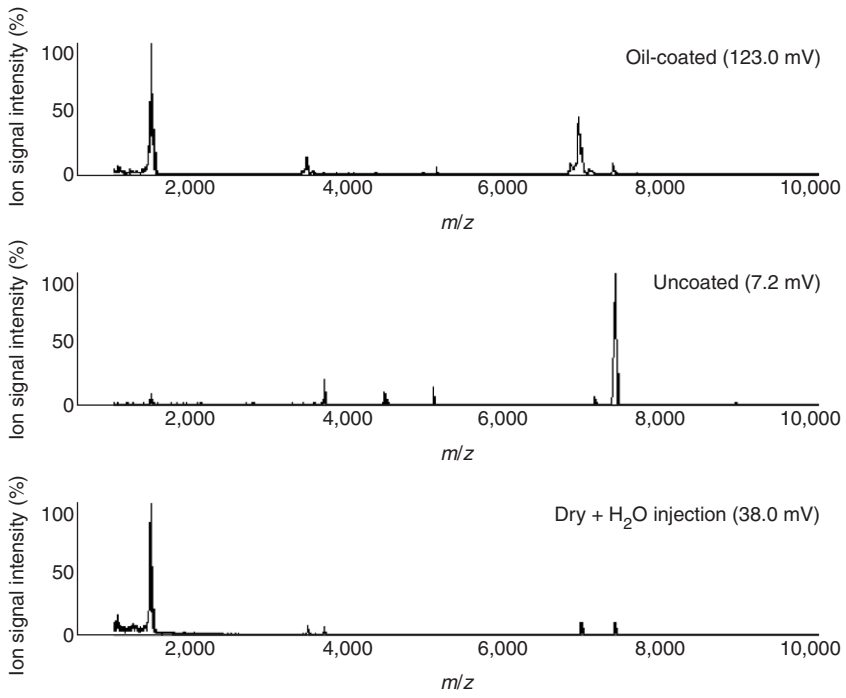


Figure 2. MALDI mass spectra of aerosolized *Bg* spores collected on oil-coated tape (top), uncoated tape (center), and uncoated tape with co-aerosolized water (bottom) obtained on a Kratos Kompact MALDI 4 mass spectrometer (Kratos Analytical, Chestnut Ridge, NY). Tape sample spots were cut and applied to a stainless-steel MALDI slide followed by a small aliquot of matrix. The sample was air-dried and inserted into the mass spectrometer for analysis. The instrument was operated in positive ion linear mode with an extraction voltage of 20 kV. Spectra presented are the sum of 50 laser shots rastered across the sample well.

the sample/matrix mixture, a plume of gaseous particles is generated and electrostatically directed into the mass analyzer.

The type and amount of the matrix compound selected directly affects the promotion of sample ions. One parameter of interest when determining the feasibility of an appropriate matrix material is the amount of laser energy required to detect sample ions. Although it is customary to increase laser power as the molecular weight of the sample increases, low-to-moderate settings are preferable for higher peak resolutions and more accurate mass measurements. Additional characteristics are low amounts of fragmentation and no or minimal matrix/sample adduct ions, which may also be an indication of the use of excessive laser power.

Certain matrix materials have been classified as superior for particular classes of compounds as well as for specific molecular weight ranges of analytes. In a field environment, however, a matrix material with a broad analyte appeal will increase detection capabilities across a wide spectrum of biological compounds. A single matrix material is preferable. The number and type of consumables are kept to a minimum because of stability concerns, contamination issues, and possible leakage and/or breakage of reagent containers during transport.

To develop an optimum sample preparation strategy, several MALDI matrices, solvent systems, and pretreatment steps (e.g., sample washing and acid extraction) were investigated. Combinations of sample treatment strategies identified for additional analysis were determined by the ability to generate high-quality spectra using several defined metrics to quantify sample quality. This evaluation was independent of a specific detection algorithm and was based on spectral qualities that make relevant signal peaks easy to detect. Factors for consideration included high signal-to-noise ratios for peaks, high ion-signal content in the mass ranges of interest, and minimal meta-stable ion fragmentation, that is, fragmentation of the molecular ion before exiting the source (ionization) region, which can result in broad peaks with low resolution and poor mass accuracy. An example is shown in Fig. 3, which compares spectra obtained with two different matrix materials, α-cyano-4-hydroxycinnamic acid (acy) and sinapinic acid (sa), dissolved in two different solvent mixtures, 1

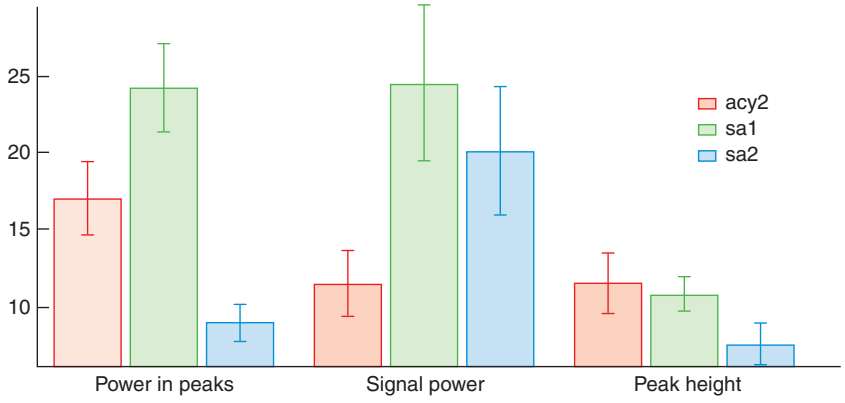
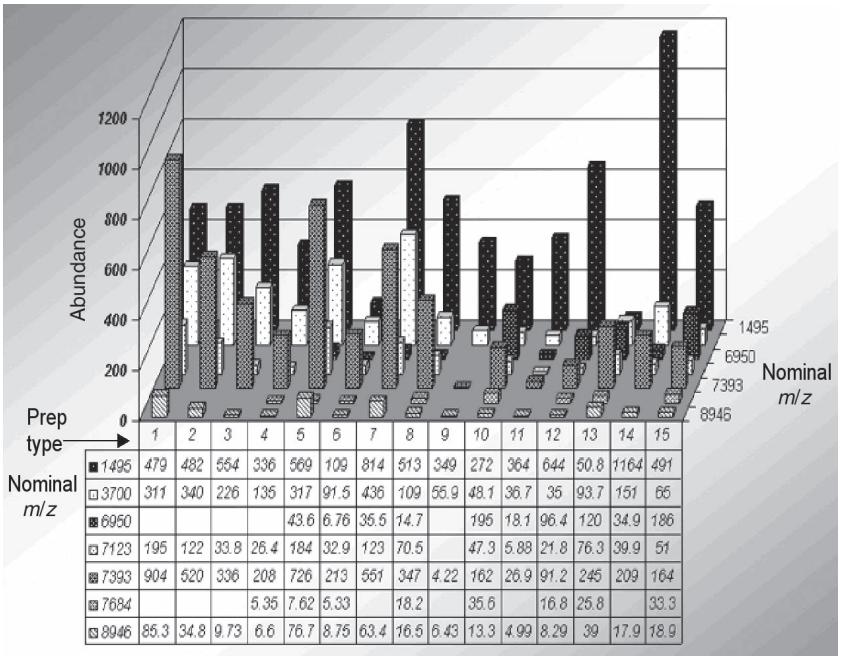


Figure 3. Comparison of matrix/solvent preparations for the mass spectral analysis of ovalbumin. Power in peaks is the sum of squared values with the target mass range after the high- and low-frequency noise have been removed, signal power is the sum of squared values with the target mass range of raw data, and peak height is the maximum value of the highest peak in the spectrum.

or 2. In the figure, power in peaks is a measure of the sum of squared values with the target mass range after the high-frequency noise and low-frequency baseline have been removed, signal power is the sum of squared values with the target mass range, and peak height is the maximum value of the highest peak in the mass spectrum.

Based on these metrics, the best data were generated using sa1. The result was not totally unexpected, since this particular matrix is better for the analysis of relatively high molecular weight compounds such as ovalbumin. A more interesting and important outcome of this analysis was the validation of acy2 as a suitable matrix for ovalbumin analysis. Molecular ions representative of ovalbumin appear at *m*/*z* 44,500 (see the toxin spectrum in Fig. 5). The acy matrix material is typically used for biological compounds with molecular weights of 20,000 Da or less, such as bacterial proteins. Varying the solvent composition used to solubilize acy extended its utility to a higher mass range, resulting in more broadband detection capabilities using a single MALDI matrix.



Nominal *m*/*z*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1495	479	482	554	336	569	109	814	513	349	272	364	644	50.8	1164	491
3700	311	340	226	135	317	91.5	436	109	56.9	48.1	36.7	35	93.7	151	66
6950					43.6	6.76	35.5	14.7		195	18.1	96.4	120	34.9	186
7123	195	122	33.8	26.4	184	32.9	123	70.5		47.3	5.88	21.8	76.3	39.9	51
7393	904	520	336	208	726	213	551	347	4.22	162	26.9	91.2	245	209	164
7684				5.35	7.62	5.33		18.2		35.6		16.8	25.8		33.3
8946	85.3	34.8	9.73	6.6	76.7	8.75	63.4	16.5	6.43	13.3	4.99	8.29	39	17.9	18.9

Figure 4. Summary of Threat Identification and Detection System (TIDS) Version 4 software detections for the MALDI mass spectral analysis of *Bg* using 15 different sample preparation protocols. Abundance (*y* axis) is shown as a function of preparation type (*x* axis) and mass line (*z* axis and table at bottom).

Another approach for signal processing incorporates Threat Identification and Detection System (TIDS) software, created to automatically detect and characterize mass spectral signature lines.[9] TIDS uses a constant false alarm rate processor to detect mass spectral peaks and reports the *m*/*z* and abundances (signal amplitudes) of those peaks. In this application, the automatic detection capability facilitated the processing of more than 5000 files containing the sample preparation trial series. Abundances of the *Bg* "biomarker" ions were considered the dependent variable, with sample preparation the independent variable in the summary graph shown in Fig. 4. As evident from this display, the majority of methods generated several characteristic *Bg* peaks. However, methods 5, 8, and 15 were shown to be superior owing to the relatively high signal intensities of detected ions over a wide mass range.

The abundance variations observed in the spectra used to generate the data in Fig. 4 can be attributed to differences in sample pretreatment techniques, such as washing a sample with water to remove interfering contaminants prior to analysis. Incorporation of an acid pretreatment step was also evaluated and integrated into the study. Two of the three methods selected for additional analysis included an acid pretreatment step in the sample preparation protocol.

SUMMARY

The current BioTOF mass spectrometer system is equipped with a sample processing module to mechanically deposit the MALDI matrix, a calibrant compound to ensure mass accuracy, and an aqueous acid solution to facilitate extraction of candidate biomarker molecules such as the small, acid-soluble proteins of *Bacillus* spores.[10,11]

Improved methods to enhance detection and identification of collected aerosols are continually being evaluated. Novel technologies are in development, such as the use of a sample preparation module to deposit chemicals in line with the collected aerosol and substrates. This not only concentrates particles to a central location but also separates out contaminants that may hinder detection.

Examples of characteristic MALDI mass spectra obtained on a commercial Kratos Kompact MALDI 4 mass spectrometer for simulant compounds of biological threat agents are shown in Fig. 5. The biomarker ions detected vary significantly in molecular weights, ranging from the cluster of peaks in the 1,400- to 1,500-Da mass range of *Bg*, a spore simulant, to the 44,500-Da molecular ion of ovalbumin in the toxin spectra. The same matrix/solvent system was used to analyze each component and highlights the universality of this combination for a multitude of substances.

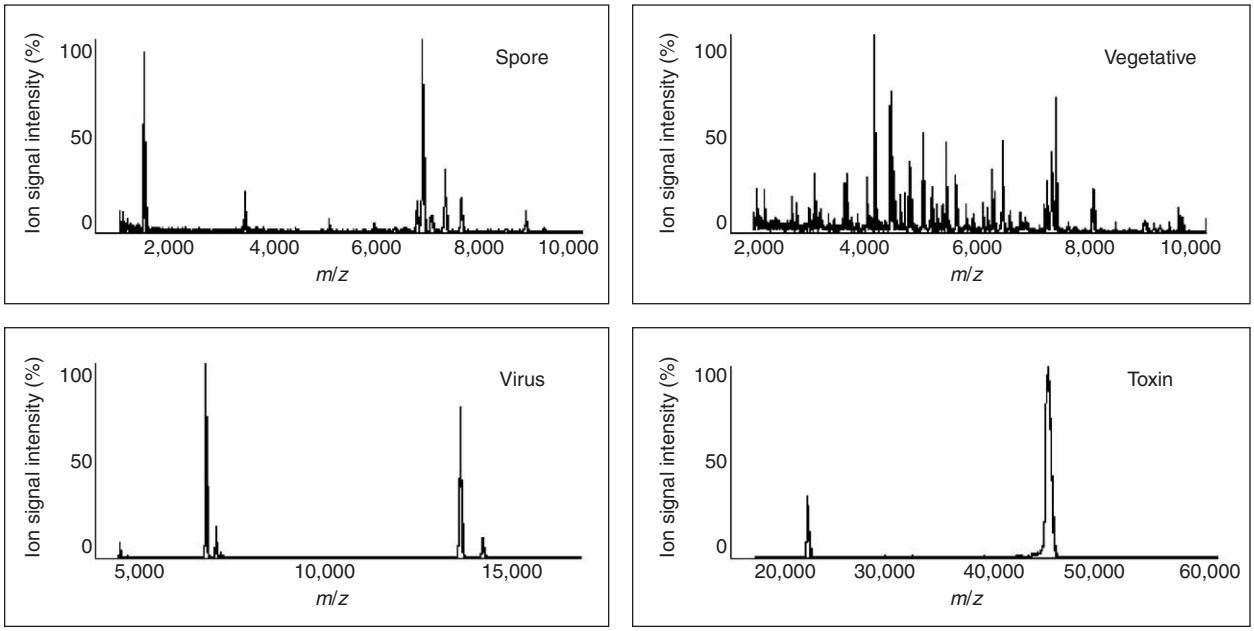


Figure 5. MALDI mass spectra of biological simulants using identical sample preparation protocols. Equal volumes of sample and matrix were applied to the MALDI sample slide and allowed to air-dry prior to insertion into the instrument for analysis. All spectra were obtained using a Kratos Kompact MALDI 4 mass spectrometer. The instrument was operated in positive ion linear mode with an extraction voltage of 20 kV. Spectra presented are the sum of 50 laser shots rastered across the sample well.

REFERENCES

[1]Fenselau, C., and Demirev, P. A., "Characterization of Intact Microorganisms by MALDI Mass Spectrometry," *Mass Spectrom. Rev.* **20**, 157–171 (2001).

[2]Lay, J. O. Jr., "MALDI-TOF Mass Spectrometry of Bacteria," *Mass Spectrom. Rev.* **20**, 172–194 (2001).

[3]van Baar, B. L., "Characterization of Bacteria by Matrix-Assisted Laser Desorption/Ionization and Electrospray Mass Spectrometry," *FEMS Microbiol. Rev.* **24**, 193–219 (2000).

[4]Bryden, W. A., Benson, R. C., Ecelberger, S. A., Phillips, T. E., Cotter, R. J., and Fenselau, C., "The Tiny-TOF Mass Spectrometer for Chemical and Biological Sensing," *Johns Hopkins APL Tech. Dig.* **16**(3), 296–310 (1995).

[5]Bryden, W. A., Benson, R. C., Ko, H. W., and Donlon, M., "Universal Agent Sensor for Counterproliferation Applications," *Johns Hopkins APL Tech. Dig.* **18**(2), 302–308 (1997).

[6]Cornish, T. J., and Bryden, W. A., "Miniature Time-of-Flight Mass Spectrometer for a Field-Portable Biodetection System," *Johns Hopkins APL Tech. Dig.* **20**(3), 335–342 (1999).

[7]Scholl, P. F., Leonardo, M. A., Rule, A. M., Carlson, M. A., Antoine, M. D., and Buckley, T. J., "The Development of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry for the Detection of Biological Warfare Agent Aerosols," *Johns Hopkins APL Tech. Dig.* **20**(3), 343–351 (1999).

[8]Anderson, C. A., and Carlson, M. A., "A Time-of-Flight Mini-Mass Spectrometer: Aerosol Collection, Capture, and Load-Lock System," *Johns Hopkins APL Tech. Dig.* **20**(3), 352–362 (1999).

[9]Hayek, C. S., Pineda, F. J., Doss, O. W. III, and Lin, J. S., "Computer-Assisted Interpretation of Mass Spectra," *Johns Hopkins APL Tech. Dig.* **20**(3), 363–371 (1999).

[10]Hathout, Y., Setlow, B., Cabrera-Martinex, R., Fenselau, C., and Setlow, P., "Small, Acid-Soluble Proteins as Biomarkers in Mass Spectrometry Analysis of *Bacillus* Spores," *Appl. Environ. Microbiol.* **69**(2), 1100–1107 (2003).

[11]Warscheid, B., and Fenselau, C., "Characterization of *Bacillus* Spore Species and Their Mixtures Using Postsource Decay with a Curved-Field Reflectron," *Anal. Chem.* **75**(20), 5618–5627 (2003).

ACKNOWLDGMENTS: The authors gratefully acknowledge the support and insightfulness of Randy Long for recognizing the complexities involved with the mass spectral analysis of biological compounds. We also acknowledge the collaborative efforts of Catherine Fenselau and her group at the University of Maryland, College Park, for their pioneering work in microorganism detection and identification.

THE AUTHORS

MIQUEL D. ANTOINE received a B.S. in chemistry from Spelman College and an M.S. in chemistry from Hampton University. She began her doctoral studies at Emory University in Atlanta, Georgia, and transferred to work with Dr. Catherine Fenselau at the University of Maryland, Baltimore County, where she received her Ph.D. in analytical chemistry. Dr. Antoine joined APL as a post-doctoral fellow in the spring of 1988 and is currently a member of the Senior Professional Staff in the Research and Technology Development Center. Her research interest centers on the use of mass spectrometry in the analysis of biological compounds. Her e-mail address is miquel.antoine@jhuapl.edu.

MICAH A. CARLSON is a mechanical engineer at APL in the National Security Technology Department's Engineering and Test Group. He received a B.S. in mechanical engineering and a B.A. in music from the University of Colorado in 1996. Mr. Carlson's specialties include structure design and analysis, materials engineering, system integration, and the development and application of miniaturized fluidics handling systems. His work areas include the development of coastal monitoring buoy systems and an aerosol collection system for use in a field-portable miniature mass spectrometer. He is currently pursuing an M.S. in mechanical engineering from The Johns Hopkins University and is a member of ASME. His e-mail address is micah.carlson@jhuapl.edu.

WILLIE R. DRUMMOND is a Senior Professional Staff engineer in APL's National Security Technology Department (NSTD). He earned his B.S. and M.A.S. degrees in management from The Johns Hopkins University in 1977 and 1989, respectively, and joined APL in 1991. Mr. Drummond has participated in major APL engineering programs, including the Navy's Transit Navigation Satellite Program, development and at-sea exercises of thermistor fluorometer conductivity chains, and collaborative biomedical programs with the JHU Medical Institutions involving laboratory experiments in brain edema, magneto-encephalography, and bio-impedance. He is a specialist in metrology; design, fabrication, and calibration of bio-impedance coils and probes; and aerosol and particle measurement. Since retiring in January 2003, Mr. Drummond has been working part-time as manager of NSTD's CTD calibration laboratory and is involved in the development of mass spectrometers. His e-mail address is willie.drummond@jhuapl.edu.

OTIS W. DOSS III received a B.A. in mathematics from the University of Virginia in 1980 and an M.S. in numerical science from the JHU G. W. C. Whiting School of Engineering in 1984. Mr. Doss has been working at APL as a resident subcontractor since 1993 and is currently a member of the Signal and Information Processing Group in the National Security Technology Department. He has been involved in developing signal processing algorithms and software for both active and passive underwater acoustic applications and is now focusing on developing graphical user interfaces for tactical display of passive underwater acoustic data. Mr. Doss is a member of the Phi Beta Kappa Society. His e-mail address is otis.doss@jhuapl.edu.

C. SCOTT HAYEK has B.S. and M.S. degrees in physics from the University of Maryland. He joined APL in 1978 and is a member of the National Security Technology Department. His work has focused on ocean physics and signal processing as well as radar signal intelligence. He has been the principal investigator and test scientist in various sea tests, studying the performance limits of passive and active sonar. Mr. Hayek now splits his efforts between ocean acoustic surveillance and counterproliferaton R&D. He is a member of the APL Principal Professional Staff and the Acoustical Society of America. His e-mail address is scott.hayek@jhuapl.edu.

ANSHU SAKSENA is a Senior Professional Staff research scientist in APL's Research and Technology Development Center. He received a B.S. in physics and mathematics from the University of North Carolina, Chapel Hill, in 1994, an M.S. in computer science from the University of Maryland, College Park, in 1996, and an M.S. in applied and computational mathematics from the JHU G. W. C. Whiting School of Engineering in 2002. Mr. Saksena joined APL in 1997 and has contributed to projects involving indoor airflow modeling, undersea warfare simulation design and development, probabilistic threat assessment for counterfeit deterrence, anomalous health event detection, modeling and detection of biological agents using mass spectrometry, and scalable learning of Bayesian belief networks. His e-mail address is anshu.saksena@jhuapl.edu.

JEFFREY S. LIN received a B.S.E. degree in mechanical/aerospace engineering from Princeton University in 1986 and an M.S. degree in computer science from The Johns Hopkins University in 1989. He is currently working on a doctorate in materials science and engineering at Hopkins. Mr. Lin is a member of APL's System and Information Sciences Group of the Research and Technology Development Center, where he develops systems for automated machinery diagnostics and nondestructive evaluation of materials. His e-mail address is jeff.lin@jhuapl.edu.