Anal. Chem. **2010,** *82,* 10203–10207

Mass Analysis of Biological Macromolecules at Atmospheric Pressure Using Nonresonant Femtosecond Laser Vaporization and Electrospray Ionization

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A nonresonant femtosecond laser pulse, with an intensity of 10^{13} Wcm^{-2}, vaporizes proteins and biomolecules intact, regardless of molecular structure, size or electronic structure for subsequent electrospray ionization and transfer into a mass spectrometer. Rapid, direct analysis from dried sample, aqueous solution and cellular material is demonstrated at atmospheric pressure using laser electrospray mass spectrometry (LEMS). Measurements are presented for lysozyme (14.3 kDa), hemoglobin from human blood, ovalbumin (45 kDa) from hen egg white and phospholipids from hen egg yolk. Mass analysis of biological material is performed without dilution, extraction or sample preparation, other than placing the biological material onto the sample plate.

Mass analysis of biological systems at atmospheric pressure is enabled by resonant laser desorption of matrix molecules such as small organic acids or water using laser pulses of nanosecond duration. The remarkable success of such methods to analyze single cells,[1] tissue,[2,3] and biological material[4,5] motivates the search for matrix-free methods for desorption to enable universal analysis in complex environments such as tissue and cells. Desorption electrospray ionization[6] (DESI) and direct analysis in real time[7] (DART) eliminate the need for matrix application, but at the expense of spatial resolution. Laser-based techniques can improve the spatial resolution but require the absorption of the laser light by the substrate or a matrix (e.g., 2,5-dihydroxybenzoic acid, sinapinic acid, water, etc.).[3,8−10] Here, we present nonresonant laser vaporization of biological macromolecules using femtosecond duration laser pulses with an intensity of 10^{13} Wcm^{-2}. This laser intensity is 7 orders of magnitude larger than conventional MALDI schemes and enables vaporization of proteins up to 45 kDa, hydrated or dehydrated, without a matrix using a laser that is not resonant (800 nm) with an electronic or vibrational transition in the sample or substrate.

The universal detection of biological macromolecules with a single technique is difficult because of the wide variety of chemical structures, polarities and low vapor pressures of the possible species. A nonresonant femtosecond laser pulse couples into all molecules at an intensity of 10^{13} Wcm^{-2} enabling vaporization of a wide range of native materials. At this laser intensity the details of the electronic structure of the target are not important and molecules adsorbed in a film, multilayer, or mixture are transferred into the gas phase intact.[11−14] Insight into the mechanism can be gleaned from early work on vaporization of cryogenic multilayers of benzene at 10^{13} Wcm^{-2} wherein only translationally energetic molecules (1−2 eV) were observed with no energy deposition into dissociation channels.[11] Thus, the femtosecond vaporization of small molecules bypasses thermal modes leading to dissociation and instead result in the transfer of intact molecules into the gas phase. Also, photographic images of leaf samples revealed significant damage around the crater for the case of nanosecond ablation in comparison to femtosecond ablation due to thermal effects.[15] We previously used laser electrospray mass spectrometry (LEMS) to demonstrate the nonresonant femtosecond vaporization of vitamin B12,[12] protoporphyrin IX,[12] peroxide-based explosives,[13] and narcotics[14] without the need for resonance or externally applied matrix.

The extension of ultrafast laser vaporization to the analysis of biological macromolecules represents a new area of research. Here we investigate the ultrahigh intensity laser vaporization and atmospheric pressure mass spectrometry for a series of macromolecules in their native states, without the addition of matrix or other material to facilitate vaporization. We investigate the non-

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10.1021/ac102409k
Published on Web 11/15/2010

resonant laser vaporization directly from cellular material, both in the (natural) liquid state and from dried systems. We first investigate the vaporization of aqueous and dehydrated lysozyme, a 14.3 kDa enzyme. We then investigate the possibility of directly analyzing hemoglobin from human blood in the liquid and dehydrated states, followed by the analysis of ovalbumin from albumen (hen egg white), and phospholipids from hen egg yolk.

EXPERIMENTAL METHODS

Sample Preparation. Pure lysozyme (Sigma, St. Louis, Missouri) and ovalbumin (MP Biomedicals LLC, Solon, Ohio) were diluted to 10^{-3} M in deionized water and 20 μL of each solution was placed on stainless steel sample slides. Chicken eggs were purchased from a local market and the albumen was separated from the yolk and a 500 μL aliquot of both the yolk and albumen were placed on separate stainless steel slides to be analyzed. Undiluted human blood (~20 μL) from a healthy volunteer was deposited directly onto a stainless steel slide. The sample slides were placed on a metal plate in the electrospray ionization (ESI) source chamber. The metal plate was supported by a three-dimensional sample stage whose translation exposes fresh sample for analysis. Each sample was analyzed in the liquid (hydrated) and the solid (dehydrated) state. The dehydrated samples, containing the same volume as the hydrated samples, were allowed to dry prior to analysis. No further sample preparation other than transfer of the sample onto the metal slide was performed.

Vaporization, Ionization, and Analysis Method. The sample was vaporized with 1.5 mJ, 70 fs laser pulses centered at 800 nm produced by a Ti:sapphire laser system (oscillator, KM Laboratories Inc., Boulder, CO, regenerative amplifier, Coherent Inc., Santa Clara, CA). The laser was focused to a spot size of ~200 μm in diameter using a 17.5 cm focal length lens, with an incident angle of 45°, with respect to the sample. The intensity of the vaporization laser at the sample was $\sim 5 \times 10^{13}$ Wcm^{-2}. The vaporized sample was captured and ionized by the electrospray plume which was emitted from the ESI needle 6.4 mm above and parallel to the sample stage. The ESI solvent contained 1:1 (v:v) methanol:water (Fisher Scientific, Fair Lawn, NJ, USA) with 1% acetic acid (J.T. Baker, Phillipsburg, NJ). The ions were then analyzed and detected using a home-built pulsed deflection orthogonal time-of-flight (TOF) mass spectrometer in a linear configuration with resolution of m/Δm of 140 at *m/z* 800. The electrospray solvent flow rate, set by a syringe pump (Harvard Apparatus), was 3 μL/min. The source setup and parameters are described elsewhere.[12−14] The resulting positive ion mass spectrum was collected and averaged for 100 laser shots (10 s) using a digital oscilloscope. *Safety Consideration: Appropriate laser eye protection was worn by all personnel, and the high voltage area was enclosed in plexiglass to prevent accidental contact with the biased electrodes. An Exposure Control Plan was approved by the University (no. 13314) for the blood analysis.*

RESULTS AND DISCUSSION

Nonresonant Laser Vaporization of Aqueous and Pure Hen Egg Lysozyme. We previously demonstrated that fragile macromolecules[12] up to the molecular weight of 1355 Da could be transferred into the gas phase using femtosecond duration laser pulses with an intensity of 10^{13} Wcm^{-2}. Proteins represent an important class of biological molecules for analysis and characterization. Lysozyme is a large biomolecule (14.3 kDa) that contains 129 amino acids and four disulfide bridges. The mass spectrum resulting from nonresonant femtosecond laser vaporization of hen egg lysozyme from aqueous solution is shown in Figure 1a. Analysis of the aqueous state is performed to simulate the natural physical state of lysozyme in the hen egg albumen. The LEMS spectrum contains primarily the 10+ and 9+ charge states. ESI mass spectra for folded proteins tend to display one or two intense peaks in comparison with the bell-shaped distribution observed for unfolded protein.[16] Furthermore, the number of charge states for folded proteins is less than that for unfolded proteins because of the restricted number of protonation sites and the pairing of acidic and basic amino acids within the folded structure.[17] The predicted charge state for the folded conformation of lysozyme is 10+, based on 20 basic sites and 10 acidic sites in the protein.[16] Previous ESI investigations reveal a similar restricted distribution at 10+, 9+, and 8+ for lysozyme under conditions that preserve the folded structure.[18−20] This measurement reveals that an intense femtosecond laser pulse (5×10^{13} Wcm^{-2}) is capable of transferring aqueous lysozyme into the gas phase without fragmentation. The charge state distribution further suggests the interesting notion that lysozyme is vaporized from aqueous solution in a folded conformation and that the intense laser pulses do not break the disulfide bonds that assist in maintaining the folded conformation.

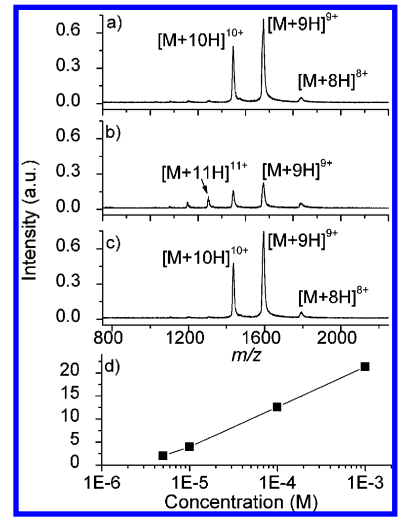

Figure 1. LEMS mass spectra of hen egg lysozyme (a) hydrated, (b) dehydrated, and (c) rehydrated. The ion signal intensity as a function of concentration can be seen in (d) for hydrated lysozyme.

The laser vaporization of pure protein from metal substrates using nanosecond duration laser pulses has been enabled through two mechanisms. The first involves the resonant absorption of the laser pulse by a matrix or the sample. The second involves absorption by the substrate leading to thermal desorption. The

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rapid heating of the metal substrate enables desorption[21] and possible unfolding of protein due to the high equilibrium temperatures reached. However, with femtosecond laser pulses the energy is deposited into the sample much faster than thermal equilibrium can be established[22] decreasing the probability of protein unfolding. In vaporizing lysozyme from aqueous solution, one could argue that the water solvent acts as a matrix for transferring the lysozyme into the gas phase, and plays a similar role to the small organics acid molecules or water typically required for desorption to occur in MALDI, MALDESI, or LAESI experiments. To eliminate small molecule matrix effects, we investigated the nonresonant laser vaporization of dehydrated lysozyme deposited onto a metal substrate. The corresponding mass spectrum for dehydrated, pure lysozyme is shown in Figure 1b. The mass spectrum contains charge states ranging from 8+ to 13+. This suggests that as the lysozyme solution dries on the metal slide, there is partial unfolding of the protein. Previous investigations[23] suggest that a secondary structural conformation transition occurs in lysozyme from α-helix to β-sheets adsorbed onto the substrate. However, when the same sample is rehydrated (20 μL of water applied to dried lysozyme) the charge state distribution returns to the folded conformation, as shown in Figure 1c. There were no low mass ions detected in these experiments which suggests that lysozyme does not fragment during vaporization. This analysis demonstrates the remarkable result that proteins can be vaporized into the gas phase intact (with no fragmentation) using laser intensities 7 orders of magnitude larger than typical nanosecond resonant excitation experiments. This implies that LEMS is a "soft" vaporization method for pure protein. Measurements without the electrospray (laser only) reveal no ion signal suggesting that the molecules are vaporized in the neutral state. DESI-like desorption is not possible during these experiments since the electrospray plume does not interact directly with the sample since it is above (6.4 mm) and parallel with the sample stage. Measurements with the electrospray only (no laser) reveal no protein signal.

The limit of detection (LOD) for the LEMS analysis of lysozyme was determined by measuring the signal intensity from serial dilutions. A 10 μL aliquot of each sample, ranging in concentration from 10^{-3} to 10^{-6} M, was deposited on a stainless steel surface and analyzed in the aqueous state. A total of three experiments consisting of 50 laser shots each can be performed on a given 10 μL sample. The resulting molecular ion intensity is plotted as a function of concentration in Figure 1d. The LOD was defined as signal-to-noise (S/N) greater than three. The LOD for this measurement occurs at a concentration of 5×10^{-6} M with a S/N = 4.4 for the 9+ charge state. This corresponds to a LOD of 17 pmol given that each experiment (50 laser shots) consumes approximately 1/3 of the sample. The corresponding LEMS mass spectra for the serial dilutions are shown in Figure S1 of the Supporting Information.

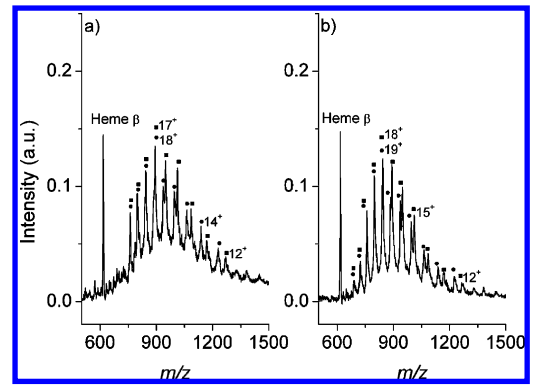

Figure 2. LEMS spectra for samples of human blood spotted on a metal surface and analyzed in the (a) hydrated, liquid state and (b) dehydrated state. Filled in squares (■) chain and filled in circles (●) represent the multiply charged α and β chains, respectively. Each spectrum was averaged over 100 laser shots for a total analysis time of 10 s.

LEMS Analysis of Blood under Atmospheric Conditions without the Aid of a Matrix or a Resonant Transition. The detection of lysozyme from the aqueous and dehydrated state using intense nonresonant laser vaporization suggests that LEMS may provide a means to analyze more complex biological systems in their native states under ambient temperature and pressure conditions. One of the most common biological fluids analyzed is blood.[24] Human blood consists of red and white blood cells, platelets and plasma. Red blood cells (40% of blood) contain hemoglobin, an oligomeric protein that has four noncovalently bound polypeptide chains with a mass of 64.5 kDa, which transport oxygen to cells. The protein contains two α chains, each composed of 141 amino acids (15 126 Da), and two β chains, each composed of 146 amino acids (15 867 Da).[25] Each chain also contains a corresponding α or β heme group. The LEMS analysis of undiluted blood in the liquid state vaporized from a metal slide is shown in Figure 2a. A bell shaped distribution of multiply charged α and β chains is observed with the maximum signal intensity at m/z 891. The LEMS spectrum of hemoglobin also shows the β heme at m/z 616, while the α heme at m/z 853 is not clearly distinguished. No fragment ions were observed in the lower mass region. This measurement reveals that proteins can be vaporized from complex biological fluids using intense femtosecond laser pulses. The observation of the multiply charged α and β subunits is consistent with control ESI measurements (Figure S2 in Supporting Information) and with published ESI data.[25] Tetramers, such as hemoglobin, have been detected using conventional ESI under mild conditions[26] where there is sufficient energy for desolvation, but not enough to cause dissociation. In the LEMS experiment, dissociation of the subunits occurs in the ESI source after capture by the acidified electrospray solvent plume. With our present apparatus, we can not determine whether intact tetramer is vaporized because the expected[27] m/z exceeds the maximum mass the hexapole can transmit in our spectrometer

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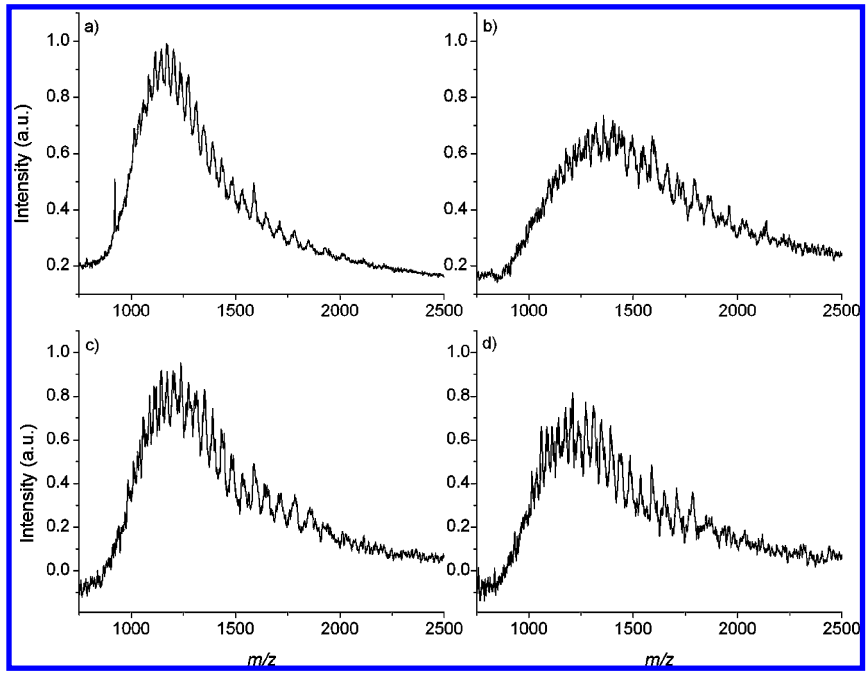

Figure 3. LEMS spectra of (a) hydrated albumen (hen egg white), (b) dehydrated albumen, (c) hydrated, and (d) dehydrated 10^{-3} M matrix-free ovalbumin. Each spectrum was averaged for 100 laser shots.

($<\sim m/z$ 3000). Blood samples have been analyzed previously using laser ablation with a 2.94 μm laser that couples into the O−H stretching mode of water[4] and using a 337 nm laser coupled to carbon powder added as a matrix in liquid blood.[28] In these studies, the intact α and β subunits were detected but the tetramer complex was not. The LEMS measurement again demonstrates that a resonant transition in the complex mixture is not required for vaporization and that no fragmentation of the α and β subunits of hemoglobin occurs during vaporization in the intense ultrafast laser field applied to the system.

To determine whether water was necessary for the nonresonant femtosecond laser vaporization process, blood was deposited and dehydrated on a metal surface. The sample was subjected to a laser pulse of intensity 10^{13} Wcm^{-2}. Once again, features indicative of hemoglobin were observed in the mass spectrum, as shown in Figure 2b. The similarity of the mass spectrum to the hydrated sample suggests that water is not necessary for the vaporization. The analysis of hemoglobin from human blood demonstrates the capability to nonresonantly vaporize, ionize, and detect large biomolecules from the native environment under atmospheric pressure and temperature with essentially no sample preparation. The total time required for the analysis is less than one minute.

Analysis of Native Cellular Material: Hen Egg White and Yolk. We next investigate the nonresonant femtosecond laser vaporization of molecules in the albumen and yolk of a hen egg. The hen egg represents cellular material[29] that, while not testing the spatially resolved detection capabilities,[14] does evaluate the capability to probe the complex environments found within a cell. Albumen (hen egg white) is composed of 90% water with the remaining 10% consisting of a mixture of glucose, vitamins and approximately 40 different proteins. The four major constituent proteins are ovalbumin (54%), ovotransferrin (12%), ovomucoid (11%), and lysozyme (3.4%).[30] The positive ion mode LEMS mass spectrum for raw albumen is shown in Figure 3a. The high m/z region of the LEMS spectrum reveals a mass spectral distribution centered at $\sim m/z$ 1175. The bell-shaped distribution has features that are attributed to multiply charged proteins. Ion signal was also detected from albumen when the sample was dehydrated, Figure 3b, and in this case the center of the distribution for the dry albumen shifts to the higher m/z region and therefore lower charge states. This experiment was performed for a variety of eggs and the data shown are representative of the set.

The most abundant protein in the albumen is ovalbumin. Ovalbumin contains a peptide sequence of 385 amino acids having a molecular weight of 42.7 kDa. The mass of ovalbumin increases to approximately 45 kDa after post-translational modification addition of carbohydrate and phosphate groups.[31] Ovalbumin has a concentration of $\sim 10^{-3}$ M in albumen and is 4−5 times more concentrated than ovotransferrin. Therefore, the signal corresponding to the bell-shaped distribution in the albumen mass spectrum is most likely from ovalbumin. To test this hypothesis, aqueous ovalbumin was analyzed at 10^{-3} M to simulate the protein's natural state in albumen. The resulting LEMS spectrum is shown in Figure 3c and d for both the hydrated and dehydrated state of ovalbumin. Again, the mass spectral distribution centered at $\sim m/z$ 1175 is observed. The distribution has a one to one mapping with the mass spectral features measured for the albumen sample, suggesting that the features observed in albumen are indeed due to ovalbumin. The calculated m/z distribution for ovalbumin along with the measured m/z values for both hydrated albumen and hydrated ovalbumin are tabulated in Table S1 in the Supporting Information and are the same within the mass accuracy of our system.

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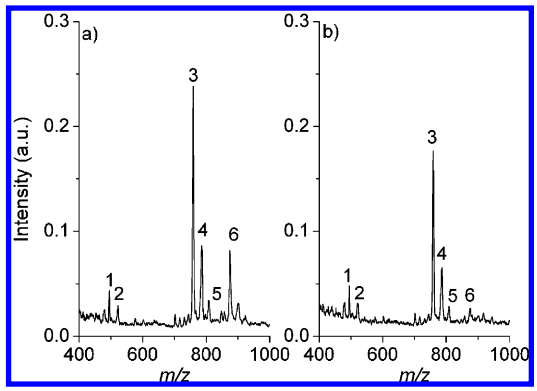

Figure 4. LEMS mass spectra of raw egg yolk (a) hydrated and (b) dehydrated. Peak 1 corresponds to $[LPC\ C16:0 + H]^+$ at m/z 496, peak 2 $[LPC\ C18:0 + H]^+$ at m/z 524, peak 3 at m/z 760 is $[PC\ 16:0/18:1 + H]^+$, peak 4 at m/z 788 is $[PC\ C18:0/C18:1 + H]^+$, and peak 5 at m/z 810 is $[PC\ C18:0/C18:1 + Na]^+$. Each spectrum was averaged for 100 laser shots.

To determine whether LEMS is capable of intracellular differentiation, hen egg yolk was deposited on a stainless steel sample slide and analyzed. Egg yolk is composed of water, carbohydrates, fat, protein, cholesterol, and phospholipids. Phospholipids make up 31% of dried egg yolk.[32] The phospholipid composition is 73% phosphatidylcholine, 15% phosphatidylethanolamine, 5.8% lysophosphatidylcholine, 2.5% spingomyelin, 2.1% lysophosphatidylethanolamine, and 0.6% inositol phospholipid.[33] The LEMS spectrum of hydrated egg yolk is shown in Figure 4a. When the yolk is placed in the sampling region, the counter current drying gas at 180 °C causes dehydration and the formation of a dehydrated "skin" layer. When the sample is left in the same XY position, the laser will drill through the dried layer to the native (hydrated) yolk, increasing the signal intensity as a function of time. The completely dehydrated yolk sample was also analyzed and is shown in Figure 4b. Peak 1 at m/z 496 and peak 2 at m/z 524 are consistent with protonated lysophosphatidylcholine (LPC) with fatty acid compositions of C16:0 and C18:0, respectively.[34] The mass spectrum displays several peaks (peaks 3−5) between m/z 760 − 810, which are consistent with a group phosphatidylcholines found in egg yolk. A similar mass spectral intensity distribution was observed in this mass range previously[34] suggesting that peaks at m/z 760, 788, and 810 are consistent with phosphatidylcholines (PC) with the following fatty acid composition: m/z 760 peak 3 $[PC\ 16:0/18:1 + H]^+$, m/z 788 peak 4 $[PC\ C18:0/C18:1 + H]^+$, and m/z 810 peak 5 $[PC\ C18:0/C18:1 + Na]^+$. The m/z feature labeled 6 was not identified but is unique to the egg yolk spectra. Egg yolk has been analyzed previously[35] using both UV (with the addition of a matrix) and IR lasers (resonant with water). LEMS employs a high intensity nonresonant femtosecond laser pulse to couple into the sample, therefore the presence of water or a matrix is not required. The LEMS analysis of albumen and egg yolk demonstrates the ability for intracellular differentiation using the same vaporization and electrospray ionization conditions. Finally, nonresonant laser vaporization of all the hydrated and dehydrated samples reported here yield comparable signal intensities. Laser-based techniques that rely on resonant transitions for desorption have reported a 100-fold decrease in signal intensity for dehydrated samples compared to hydrated samples.[8]

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CONCLUSIONS

We demonstrate a new release technology for hydrated and dehydrated biological materials based on nonresonant laser vaporization using an 800 nm, 70 fs laser pulse with an intensity of 5×10^{13} Wcm^{-2}. The nonresonant vaporization occurs without the addition of a matrix. Once vaporized, the molecules were ionized, analyzed and detected using electrospray ionization time-of-flight mass spectrometry. We have demonstrated that the nonresonant femtosecond laser vaporization method is capable of delivering biological macromolecules, ranging from phospholipids to enzymes and from pure solids to complex biological fluids, into the gas phase, intact. This analysis does not require homogenization of tissue samples or further sample preparation. Vaporization and ionization of a variety of biological molecules under ambient pressure and temperature conditions allows for rapid mass spectral analysis since sample preparation and laser-sample (or substrate) resonance is eliminated. Finally, we note that with the rapid advances being made in solid state laser technology, the size and cost of femtosecond lasers is decreasing. Presently, turn key lasers are available that are on a size and cost scale of commercial electrospray systems making them affordable for vaporization methods.

ACKNOWLEDGMENT

This work was supported by the Army Research Office W911NF0810020. The apparatus was constructed with support from National Science Foundation CHE0518497.

SUPPORTING INFORMATION AVAILABLE

LEMS mass spectra for the serial dilutions used in calculating the LOD, ESI mass spectrum of human blood and table of calculated and measured m/z values for hydrated albumen and hydrated ovalbumin. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review September 9, 2010. Accepted October 22, 2010.

AC102409K