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Laser ablation sample transfer for mass spectrometry

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LASER ABLATION SAMPLE TRANSFER FOR MASS SPECTROMETRY

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

3D	three-dimensional
CAN	acetonitrile
CCA	α-cyano-4-hydroxycinnamic acid
CE	capillary electrophoresis
DART	direct analysis in real time
DESI	desorption electrospray ionization
DHB	2,5-dihydroxybenzoic acid
ELDI	electrospray assisted laser desorption ionization
ESI	electrospray ionization
HPLC	high-performance liquid chromatography
IR	infrared
IR-LAMICI	infrared laser ablation metastable-induced chemical ionization
ITO	indium tin oxide
LAESI	laser ablation electrospray ionization
LA-FAPA	Laser ablation with a flowing atmospheric pressure afterglow
LA-ICP	Laser ablation inductively coupled plasma ionization
LC	liquid chromatography
LDI	laser desorption ionization
LEMS	laser electrospray mass spectrometry
LESA	liquid extraction surface analysis
LMJ-SSP	liquid microjunction surface sampling probe
m/z	mass-to-charge ratio
MALDESI	matrix-assisted laser desorption electrospray ionization
MALDI	matrix-assisted laser desorption/ionization
MS	mass spectrometry
NALDI	nanostructure assisted laser desorption ionization
NMR	nuclear magnetic resonance
OPO	optical parametric oscillator
PC	phosphatidylcholine
PVA	poly(vinyl alcohol)
QIT	quadrupole ion trap
RSD	relative standard deviation
S/N	signal-to-nose
SA	sinapinic acid
SIM	selected ion monitoring

TFA	trifluoroacetic acid
TIC	total ion current
TIE	total ion electropherogram
TLC	thin layer chromatography
TOF	time-of-flight mass spectrometer

ABSTRACT

In this research, a new ambient sampling technique for mass spectrometry was developed that uses an infrared laser to ablate materials under ambient conditions that are captured in a solvent or on a surface. An infrared optical parametric oscillator (OPO) laser system at 3 μm wavelength was focused onto samples for ablation at atmospheric pressure. The ablated materials were transferred to a solvent or surface. For off-line electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) analysis, the ablated material was captured in a static solvent droplet that was deposited on a MALDI target or flow-injected into a nanoelectrospray source. The direct analysis of biological fluids for off-line MALDI and electrospray was demonstrated with untreated blood, milk, and egg. For one-line ESI, the ablated material was captured in an exposed flowing solvent stream that carried the ablated material to the ESI source. For on-line liquid chromatography ESI (LC-ESI) and on-line capillary electrophoresis ESI (CE-ESI), the ablated material was captured in the flowing solvent and injected into a LC column or a capillary with pressure driven or electrokinetic flows, respectively. The performance of the system was assessed using peptide and protein mixtures ablated from the target and analyzed with LC or CE separation. For MALDI imaging with IR laser ablation sampling, a thin tissue section placed on a microscope slide was scanned in two dimensions under a focused IR laser beam to transfer material to the target slide via ablation. After the material was transferred to the target slide, it was analyzed using MALDI imaging. Images were obtained from peptide standards for initial optimization of the system and from mouse brain tissue sections.

CHAPTER 1. INTRODUCTION*

Quantitative and qualitative analysis of chemical compounds from biological samples has played an important role in biological, medical and clinical studies aimed at understanding cell function, disease states, and in drug development.[5-7] Assessment of biochemical changes in samples provides important clues for diagnosis, the extent of the disease process, designing therapies, and evaluating the efficacy of treatment.[8,9]

A number of analytical methods have been introduced to study biological systems, for example fluorescence,[10,11] infrared (IR),[12] Raman,[13-15] nuclear magnetic resonance (NMR) spectroscopy,[16,17] electron microscopy,[18] atomic force microscopy,[19] and electrochemical biosensors.[20,21] Mass spectrometry (MS) is one of the most successful and popular techniques for the analysis of chemical compounds due to high sensitivity and universality.[5,9,22] Mass spectrometry was introduced in the early 20th century and has been used in different areas such as pharmaceutical science, petroleum industry, forensic science, and biological science.[23-28]

One of the main challenges in biological mass spectrometry is the detection chemical species with low abundance in biological samples due to large number of individual components present in the samples.[29-31] Over the past several decades, various approaches for analysis of the biological sample coupling separations with mass spectrometry have been introduced.[32,33] A commonly used method is the separation of the chemical compounds by liquid chromatography (LC)[34-37] or capillary electrophoresis (CE)[38-41] and subsequent analysis by MS after sample preparations such as centrifugation, sonication, extraction and pre-concentration.[42-45] The

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coupling of separation techniques with MS can significantly enhance dynamic range.[46-48] However, one of the limitations of the approach is that the sample preparation can be labor-intensive and time-consuming.[42-45,49]

In recent years, MS with direct sampling ionization under atmospheric conditions have been introduced.[50-52] This technique is called ambient MS. With this technique, MS is able to analyze samples in their native environment with little or no sample preparation.[53] Due to its abilities for direct and rapid analysis, ambient MS has been attractive for biological analysis in biological fluids and tissues.[54-56] One of the attractive applications of ambient MS is tissue imaging. Imaging MS can detect chemical compounds, including drugs, metabolites, lipids, peptides, and proteins, with sub-millimeter spatial resolution, and can obtain qualitative information on the compounds in tissues without chemical label or staining.[57-59]

The goal of the research described in this dissertation was to develop an ambient sampling technique for mass spectrometry that can be coupled both with separation techniques and imaging for biological sample analysis. The approach is based on IR laser ablation sample transfer. A laser-based approach gives good spatial resolution in comparison to spray or liquid surface sampling methods because the laser has a smaller spot size and there is no solvent diffusion into the sample.[50] Compared with UV laser sampling, the IR laser removes a larger quantity of material and allows matrix-free operation.[60] In addition, the deeper penetration of the IR laser enables removal of deeply embedded analytes in materials such as thin layer chromatography plates, polyacrylamide gels, and tissue.[61,62] Capturing the ablated material in a solvent or on a surface allows further processing prior to mass spectrometry, for example separation by chromatography or electrophoresis.

1.1 Ambient Mass Spectrometry

Ambient mass spectrometry is the direct analysis of materials in their native environment with ions typically created outside the mass spectrometer.[50-55,63] With ambient methods, sample preparation is minimal and, in some cases, unnecessary. Due to its capabilities for fast direct analysis, ambient mass spectrometry has important applications in forensics, environmental analysis, and medical diagnostics.[54,63,64] The rapid growth of ambient mass spectrometry has been led by the two techniques of desorption electrospray ionization (DESI)[65] and direct analysis in real time (DART),[66] which form ions by directing charged droplets or ions, respectively, at the sample of interest.

A schematic of DESI is shown in Figure 1-1. In DESI, a fine spray of solvent charged droplets from an electrospray ionization source is directed at a sample surface and forms a thin solvent film on the sample surface. The thin solvent film extracts some of the chemical

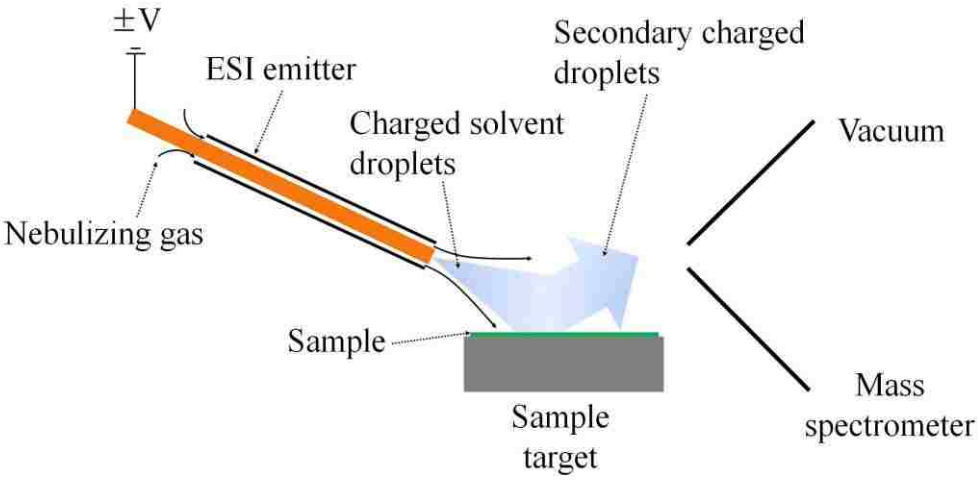

Figure 1-1. Schematic of desorption electrospray ionization (DESI).

compounds that are subsequently released to secondary charged droplets containing the extracted compounds by the shear forces of incoming spray. As the secondary droplets move through the atmospheric pressure interface into the mass spectrometer, ionization occurs following

electrospray ionization (ESI) mechanisms from the secondary droplets; the size of the secondary charged droplets are continuously reduced by evaporation of the solvent and produce gas-phase ions.[67] A schematic of DART is shown in Figure 1-2. With DART, the sample is bombarded by metastable ions from a glow discharge combined with thermal energy transfer to the sample surface. The sample is thermally desorbed and interacts with the metastable ions to be ionized.

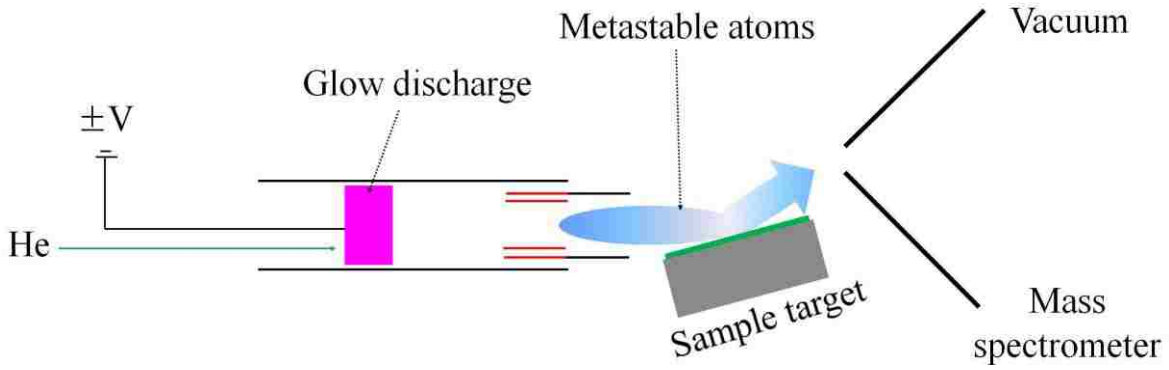

Figure 1-2. Schematic of direct analysis in real time (DART).

Ambient surface analysis can also be achieved by direct liquid extraction. This technique extracts analytes from the sample surface by contact of a liquid microjunction with the sample surface. Ambient MS techniques based on direct liquid extraction includes liquid extraction surface analysis (LESA),[68] liquid microjunction surface sampling probe (LMJ-SSP),[69] and nano-DESI.[70,71] In LESA, as shown in Figure 1-3, a pipette tip containing the extraction solvent creates a liquid junction between the tip and the sample surface by dispensing a specific volume of the extraction solvent, and then the solution containing the dissolved sample is aspirated back into the tip. The solution is sprayed through a nanospray tip for mass spectrometric analysis by applying the appropriate high voltage.[68] LMJ-SSP uses a solvent flow to extract the analytes from a sample surface in which a liquid microjunction is formed between the end of the probe and the sample surface.[69,72] The LMJ-SSP assembly consists of a dual coaxial tube. The outer

tube continuously supplies a fresh extraction solvent and creates the liquid microjunction between the probe and the sample surface to extract materials from the sample. The inner tube is used to deliver the solution containing the extracted materials to an ESI source for MS analysis. Nano-DESI uses two fused silica capillaries attached at a solvent bridge formed on the sample surface. One capillary supplies solvent to create and maintain the liquid bridge, while the second capillary transports the extracted materials from the bridge to an ESI source for MS analysis.[70,71]

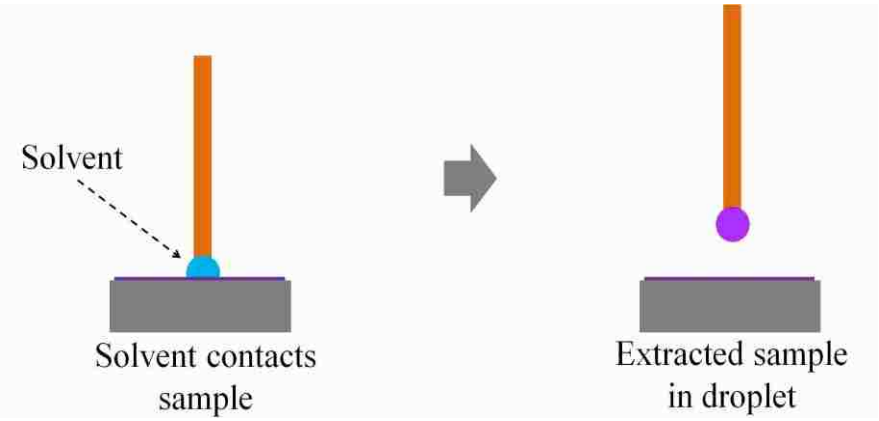

Figure 1-3. Schematic of liquid extraction surface analysis (LESA).

Ambient surface analysis using direct liquid extraction has been demonstrated for the analysis of biological samples, including tissue[73-76] and dried biological fluids.[77-79] These approaches offer some potential advantages as compared with DESI. One of the advantages is improved sampling efficiency without the worry of droplet scattering occurring when the nebulizing gas hits the solvent layer on the sample surface formed by previous charged solvent droplets.[70,80,81] The other advantage is the decoupling of the sampling from the ionization that allows liquid separation between the sampling and an ionization source.[75]

Lasers can be used to remove material from a sample and deliver it to the electrospray or other source for ionization, eliminating the need for charged droplet or metastable ion interaction

with the surface. The general concept for laser-based ambient mass spectrometry is shown in Figure 1-4. Laser ablation sample transfer to an electrospray source can be accomplished either with or without a matrix in analogy with laser desorption ionization (LDI; without a matrix) and matrix-assisted laser desorption ionization (MALDI; with a matrix). The laser-plus-electrospray method was first demonstrated in 2005 and used a 337 nm laser and no matrix.[82] With this approach, the UV laser is directed at the surface and ions are created when the plume of desorbing material interacts with the electrospray. The authors called this technique electrospray assisted laser desorption ionization (ELDI). ELDI has been used for the analysis of a wide range

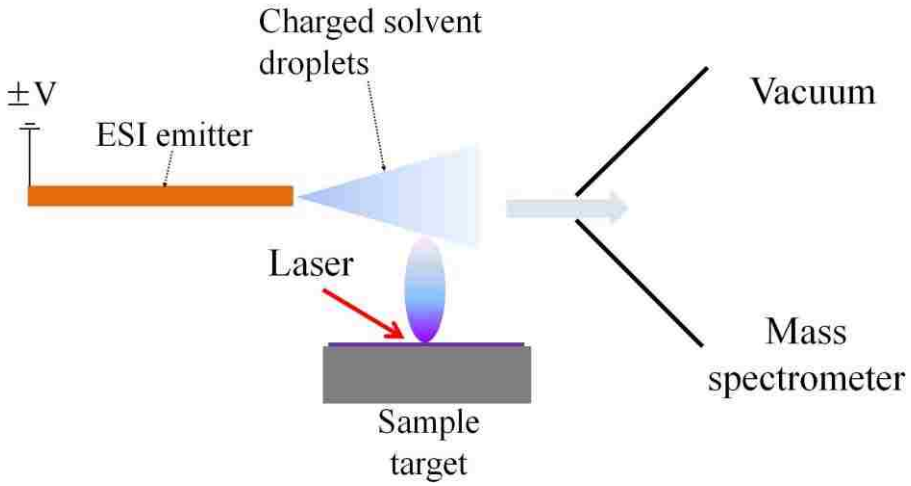

Figure 1-4. Schematic representation of laser-based ambient mass spectrometry.

of biological samples in the dried or wet state, including blood, serum, tears, whole cow milk, and tissue samples.[83,84] A second variant of laser desorption combined with ESI used a 337 nm UV laser and a MALDI matrix to aid in desorption, but not necessarily in ionization. This technique was called matrix-assisted laser desorption electrospray ionization (MALDESI).[85] An approach using a non-resonant femtosecond laser, called laser electrospray mass spectrometry (LEMS), has recently been reported[86] and demonstrated for spatially resolved ambient mass

spectrometry.[87]

Discharge sources can be used to ionize the removed materials by laser desorption or ablation instead of an ESI source. Laser ablation inductively coupled plasma ionization (LA-ICP) uses a high temperature plasma to which the removed materials are delivered by a carrier gas, such as argon or helium.[88] Laser ablation with a flowing atmospheric pressure afterglow (LA-FAPA) uses metastable ions to ionize the removed material by a 266 nm UV laser.[89] In infrared laser ablation metastable-induced chemical ionization (IR-LAMICI),[90] IR ablated materials from the sample surface were interacted with the metastable plume and ionized within the plume by gas-phase chemical ionization. These methods are limited to atoms (ICP) and small molecules.

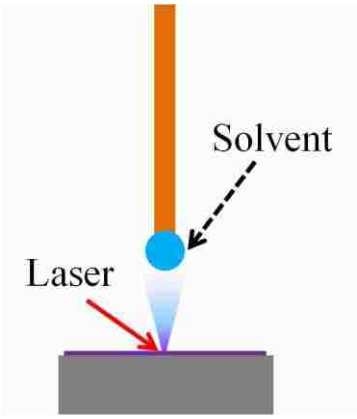

Figure 1-5. Schematic representation of laser ablation sample transfer to a solvent.

Ablation and ionization can be decoupled if the material is captured and subsequently ionized as shown in Figure 1-5. The transfer of material with a laser can be done with negligible degradation of the biological material. For example, double-stranded DNA molecules up to 1000 base pairs can be ablated and captured on a target with no fragmentation,[91,92] and ablated proteins can be used to create thin films that retain the activity of the component biological molecules.[93] Recently, Huang et al. used a near-IR (1064 nm) pulsed laser to desorb and ablate material that

was captured in a droplet. The droplet was deposited on a target and mixed with matrix for a standard MALDI analysis. Proteins could be transferred this way with little to no fragmentation.[94] Ovchinnikova et al. used a similar droplet capture approach coupled to an electrospray mass spectrometer.[95] A 532 nm visible laser was used to ablate proteins and other molecules that were captured in a solvent droplet and flow injected into the electrospray source with no fragmentation observed in the mass spectra.

1.2 IR laser-based Ambient Mass Spectrometry

Lasers operating in the mid-IR wavelength region near 3000 nm wavelength have been used for MALDI,[96] but applications have been limited due to the large quantity of material ejected that is not compatible with commercial MALDI instruments operating at vacuum and with high extraction fields.[97,98] In contrast, the greater material removal of IR lasers is an advantage for ambient ionization techniques.[99-101] The use of an infrared laser to desorb or ablate material for ionization in an electrospray was first reported in June of 2007,[102] and has been referred to using several different acronyms including laser ablation electrospray ionization (LAESI)[100] and infrared matrix-assisted laser desorption electrospray ionization (IR-MALDESI).[99,101] The volumetric energy deposition with a mid-IR laser is comparable to that of UV lasers, but the depth of laser penetration is an order of magnitude larger.[60] The greater penetration depth of IR lasers can be an advantage in removing deeply embedded materials such as biomolecules in polyacrylimide gels[61,103] and in tissue samples.[62] The mid-IR laser can be also operated without externally added matrix because water contained in the biological sample serves as effective laser desorption matrix.[104] Another advantage of mid-IR lasers is that the characteristics of the ablation plume can be widely varied simply by changing the IR laser

wavelength in a wavelength tunable system.[105,106] This tunability of the ablation plume characteristics gives a greater level of control in the quantity of material removed. The IR laser-based ambient mass spectrometry approaches have been demonstrated for the analysis of a variety of biomolecules, including metabolites, lipids, carbohydrates, and proteins in biological fluids and tissue samples.[101,107,108]

1.3 Coupling Separations with Mass Spectrometry

Ambient ionization approaches have been coupled to a variety of separation methods. DESI has been coupled to thin layer chromatography (TLC) by directing the spray at the separation bands on a TLC plate.[109-111] Reading TLC plates with DART can be accomplished in a similar manner.[112] With ELDI, the material on the separation band of the TLC plate is removed by a pulsed laser and then ablated sample interacts with electrospray charged droplets to form ions.[113] The coupling of high-performance liquid chromatography (HPLC) to DESI[114] and DART[115] can be accomplished by directing the spray or metastable beam to the HPLC effluent at the tip of a fused-silica capillary. MALDESI has been coupled to flow reactors in which the laser is directed at the effluent exiting a capillary tip.[116] Visible wavelength laser ablation with capture in a suspended liquid droplet has also been coupled with LC.[95]

The combination of capillary electrophoresis (CE) with ambient mass spectrometry has also been reported. An off-line CE interface to DESI has been demonstrated using a rotating Teflon disk covered with paper.[117] In this experiment, the samples were separated in a CE column and the temporal separation of the eluting analytes from the capillary column was encoded onto the paper disk. After separation and deposition, the surface was analyzed by DESI. Desorption electrospray ionization can also be used to ablate liquid chromatography effluent.[114]

The use of DART for on-line coupling to liquid chromatography[115] and to CE[118] has been demonstrated using a spray interface. In both cases, the effluent is nebulized and anaytes were directly ionized by DART and sampled into the mass spectrometer for detection. Continuous flow MALDESI has been demonstrated as an on-line reaction monitoring system.[116]

1.4 Imaging Mass Spectrometry

Many ambient ionization methods have been used for mass spectrometry imaging.[119] DESI has been used to obtain images that indicate the distribution of drugs in rat tissue sections[120] and mouse whole body sections.[121] The related technique nano-DESI, that uses a combination of contact sampling and nanoelectrospray, was used to image rat brain and human kidney tissue sections at a spatial resolution of 12 μm.[73] Laser desorption and ablation can also be used to improve the spatial resolution of ambient imaging. For example, IR laser desorption coupled with electrospray has been used to image single plant cells.[122] Laser ablation coupled with plasma chemical ionization has been used to image drug samples,[90] and laser ablation inlet ionization was used to image mouse brain tissue under ambient conditions.[123] Laser irradiation directed at the back side of a sample target is known as transmission geometry, and this ablation mode has been coupled with solvent capture for chemical imaging.[95] Transmission mode ablation transfer of has been coupled off-line with MALDI imaging of biomolecules in tissue.[4] Although the ability to image under ambient conditions is a powerful analytical tool, it is limited to the observation of the most abundant compounds in the sample. The ideal ambient imaging approach is one that couples a separation step between the sampling and the mass spectrometer ion source.

1.5 Objectives

The overall goal of this research was to develop an IR laser-based ambient sampling technique for mass spectrometry that can be coupled both with separations and imaging for biological sample analysis. To achieve this goal, the specific components of the project were 1) to develop IR laser-based ambient sampling and apply it to off-line ESI and off-line MALDI; 2) develop interfaces for on-line ESI and couple to on-line liquid chromatography (LC); 3) develop an interface for on-line capillary electrophoresis (CE); 4) apply laser ablation sample transfer to MALDI imaging.

The first set of experiments, described in Chapter 3, was designed to demonstrate IR laser ablation sample transfer using static solvent droplet capture for off-line MALDI and off-line ESI.[1] The second set of experiments, described in Chapter 4, was designed to develop the interfaces for coupling laser ablation sample transfer to on-line ESI and to on-line LC-ESI.[2] In this system, the IR ablated material was transferred into an exposed flowing solvent stream that carried it to the ESI source. Post-ablation separation was accomplished using a capillary column downstream of the capture zone. The third set of experiments, described in Chapter 5, was aimed at developing an interface for coupling laser ablation sample transfer to on-line capillary electrophoresis (CE).[3] In this system, the IR ablated material was captured in the exposed sampling solvent and then loaded into CE separation capillary by electrokinetic injection. The separated materials were ionized using an ESI source with a liquid junction interface. The fourth set of experiments, described in Chapter 6, was an application of laser ablation sample transfer to MALDI imaging.[4] In this system, a thin tissue section on a sample slide was irradiated in transmission mode under a focused IR laser beam to transfer materials to a MALDI or nanostructure assisted laser desorption ionization (NALDI) target. After the material was

transferred to the MALDI or NALDI target, it was analyzed using MALDI imaging using a time-of-flight (TOF) mass spectrometer.

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CHAPTER 2. INSTRUMENTATION

This chapter contains a description of the instrumentation used in this research. A brief overview of mass spectrometry, time-of-flight mass spectrometers, quadrupole ion trap mass spectrometers and detailed information on each experimental configuration is presented. System parameters during typical mass analysis are provided for each project with more detailed materials and methods discussed in later chapters.

2.1 Mass Spectrometers

Mass spectrometry (MS) is one of the most powerful analytical techniques due to its high sensitivity and universality[1-3] for analysis of chemical compounds in a sample by measuring the mass-to-charge ratio (*m/z*).[4] This technique offers information on chemical structure and relative abundance. After the first mass spectrometers were introduced in the early 20th century, these instruments have been used in different areas such as pharmaceutical science, petroleum industry, forensic science, and biological sciences.[5-10] Mass spectrometry has been increasingly used in the study of biological systems, including metabolomics,[5] lipidomics,[6,10] peptidomics,[7] and proteomics[7,8] and become an essential technique in the field of biological study. These advances have been led by two ionization techniques, electrospray ionization (ESI)[11-13] and MALDI,[13,14] coupled with various mass analyzers. In recent years, mass spectrometry has been extended to ambient sample analysis.

2.2 Ion Trap Mass Spectrometer

The three dimensional quadrupole ion trap (3D QIT) can be used as a mass spectrometer.[15] The ion trap consists of three electrodes as shown in Figure 2-1. Two of the three electrodes are end-cap electrodes and the other is ring electrode. One end-cap electrode has a

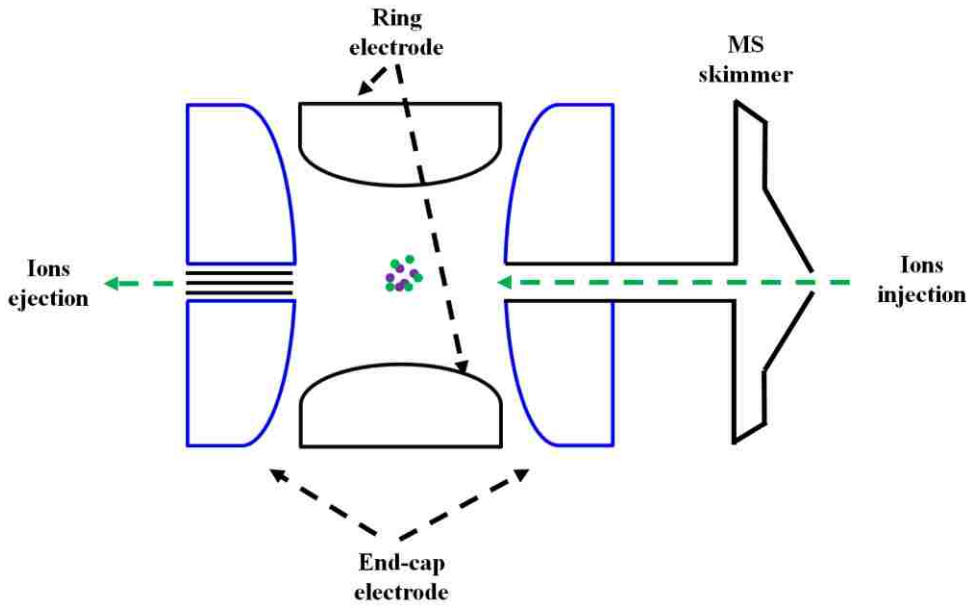

Figure 2-1. Schematic of three dimensional quadrupole ion trap.

single small central aperture through which ions can enter the trap and the other has several small apertures in the center through which ions exit the trap for detection. The third electrode is a ring electrode positioned between these two end-cap electrodes. All of the electrodes are of hyperbolic geometry.[16] With a QIT, mass separation is achieved by trapping the ions in the space between the three electrodes at which sinusoidal and static voltages are applied to the ring electrode and the end-cap electrodes, respectively, to generate electric fields resulting in and trapping ions in stable oscillating trajectories. The motion of the trapped ions is dependent on the applied voltages and their individual mass-to-charge (*m/z*) ratios. To detect the ions in the trap, the voltage applied to a ring electrode is changed to make the trajectory of the ions unstable

which results in their ejection through the end-cap. Ions are ejected as a function of the mass to charge ratio and by scanning the voltage, a mass spectrum for the trapped ions can be produced.

The ion trap mass spectrometer used in this work was a Hitachi M8000 3DQ ion trap

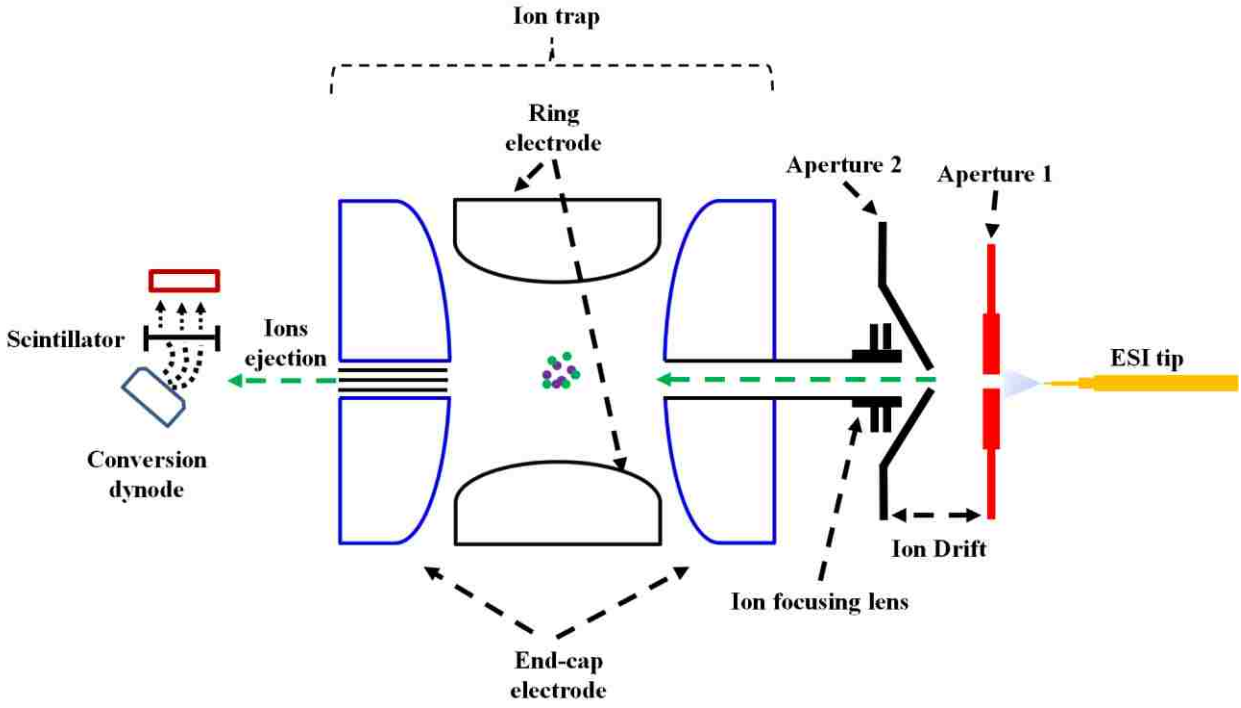

Figure 2-2. Schematic of the Hitachi M8000 3D QIT mass spectrometer.

mass spectrometer is equipped with an ESI source, a three dimensional quadrupole ion trap (3D QIT) mass analyzer, and a conversion dynode detector. A schematic of the instrument is shown in Figure 2-2. Ions generated from the ESI source enter the ion trap through aperture 1, aperture 2, and an ion focusing lens. The temperatures of aperture 1, and aperture 2 were at 180 ℃ and 120 ℃, respectively. The ion drift voltage and ion focus voltage were set to 100 V and 30 V, respectively. The trapped ions were ejected into the photomultiplier conversion dynode detector. Ions were accumulated for 100 ms in the trap and each mass spectrum was averaged for 2 min. The maximum mass range of Hitachi M8000 ion trap mass spectrometer is 2000. To perform

nano-ESI analysis, the original ESI source on the ion trap was replaced with a nanoelectrospray emitter. The nanoelectrospray emitter was 360 µm OD × 50 µm ID with a 15 µm tip ID (SilicaTip, New Objective, Woburn, MA, USA).

2.3 Time-of-Flight Mass Spectrometry

A time-of-flight mass spectrometer (TOF MS) is a mass analyzer in which mass-to-charge ratio (*m/z*) of ion is determined by measuring the flight time of ions in a field-free region.[17] One of the advantages of TOF over other mass analyzers is that there is no need to scan because all ions are detected at once. The TOF MS is composed of an ion source, an acceleration region, a field-free drift region and detector that are placed in a vacuum chamber. Ions are accelerated into the field-free drift region. The acceleration potential gives all of the ions the same kinetic energy if differences in initial positions and velocities are the same. In the field-free drift region ions, different mass-to-charge ratios travel at different velocities and arrive at the different times at the detector; the lighter ions travel faster and arrive at the detector first.

The two types of TOF mass analyzers most often used are linear and reflectron (ion mirror). A schematic of a linear TOF MS system is shown in Figure 2-3. The accelerated ions enter the field-free flight tube and move to the detector at the end of the flight tube. Ions with different *m/z* traverse the field-free region with different velocities and reach the detector at different times. From the flight time, the *m/z* of the ion can be calculated.[18]

The conditions of the ions at the point of ionization affect the resolution of linear TOF MS. Even though ions with the same mass enter the field drift region, they can reach the detector at different time due to different initial velocities and positions, resulting in lower resolution.[19] Delayed ion extraction and the reflectron have been used to maximize the resolution. In delayed

ion extraction,[20,21] ions are extracted from the ion source after some short time delay (a few hundred nanoseconds) following ion ejection from the sample (for example by laser irradiation). Ions with lower velocity are accelerated more and the ions with higher velocity are accelerated less when the extraction potential is applied because ions with a higher initial velocity are farther from the extraction grid but slower ions are closer to the extraction grid. A proper setting of the delay time focuses ions with the same *m/z* but different initial kinetic energy to reach the detector at the same time.

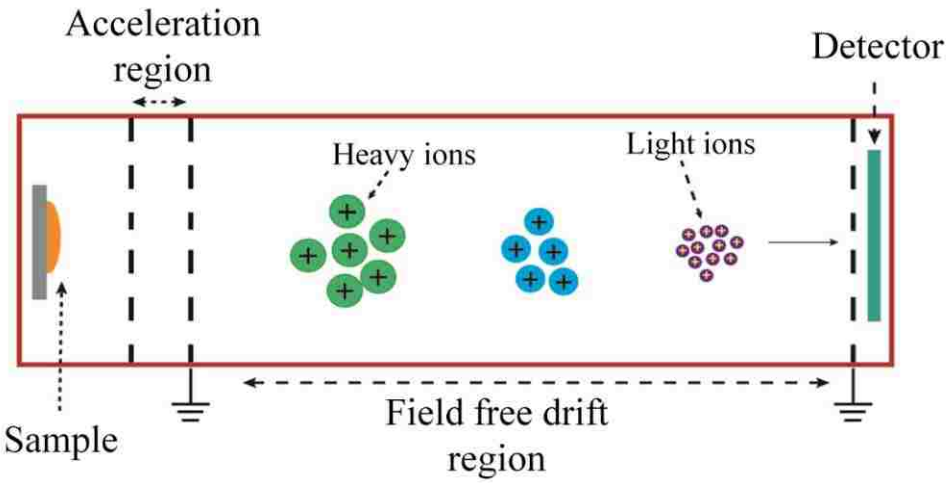

Figure 2-3. Schematic of a linear TOF mass analyzer.

Another technique to improve resolution is the reflectron that is an energy-correcting device that minimizes the effects of initial ion kinetic energy spreads.[22] Figure 2-4 shows a single stage reflectron. The reflectron is an electrostatic mirror composed of a series of ring electrodes. The electrodes are held at a repelling potential. In reflectron TOF, ions with higher kinetic energy (but the same *m/z*) have a longer flight path because the ions penetrate deeper into the reflectron field. Ions with lower kinetic energy have a shorter flight path because the ions do not penetrate as far into the reflectron. Thus, with a proper setting of ring voltages, the longer flight

time of the faster ion in the drift regions is compensated by extra time in the mirror. Longer flight path length (L1+L2) is another factor that can improve mass resolution.[23]

The Bruker Ultraflextreme TOF mass spectrometer used in this work is equipped with a frequency tripled Nd:YAG 355 nm solid-state laser with a homogenized modulated beam (Smartbeam II) laser operating 1 kHz to desorb a material from the sample deposited on a target.[24] The laser spot size is controlled with a computer at a diameter from 10-100 µm. The MALDI-TOF mass spectrometer can be operated in reflectron or linear modes. The resolution of this system is 40,000 in the *m/z* range from 700 to 5000, and the mass accuracy is 1 ppm and 5 ppm for internal and external calibration, respectively.

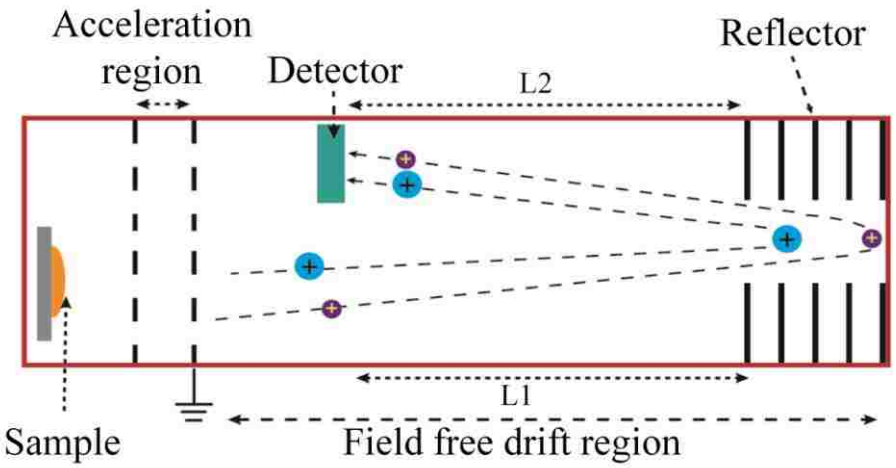

Figure 2-4. Schematic of a reflectron TOF mass analyzer.

Several targets or adapter plates for imaging slides and NALDI targets can be used with this system. For off-line MALDI, a target with 387 deposition spots was used on which the ablated material was deposited. For off-line MALDI imaging, indium tin oxide (ITO) coated microscope slides (259387, Bruker Daltonics, Billerica, MA, USA) or NALDI target (252248,

Bruker Daltonics, Billerica, MA, USA) were used. The MALDI plate or adapter plate can be automatically read by the mass spectrometer upon insertion due to a specific target transponder.

This instrument has been used with different types of samples such as bacteria,[25] blood serum, and plasma.[26] In addition, the MALDI-TOF can be used to image peptides and proteins in tissue. For tissue imaging, individual mass spectra are obtained at regular intervals over the tissue. The spatial distribution and intensity of each mass peak obtained in the mass spectra are visualized by color as a function of its position on the tissue surface.

2.4 Laser Ablation Sample Transfer

To perform laser ablation sample transfer, samples deposited on targets were irradiated with a pulsed infrared optical parametric oscillator (OPO). An OPO consists of non-linear crystals in an optical cavity.[27] A high intensity laser beam (pump) at a certain frequency is directed into the cavity containing the non-linear crystals. As a result of the interaction between the laser radiation and the crystals, the incoming laser light is split into two beams, collinear with the input, that have energies that sum to that of the incoming beam. The wavelengths of these beams can be tuned by changing the angle of the crystals with respect to the laser beam. Various tuning ranges can be achieved by properly selecting the laser, the non-linear crystals, and the optical components.

The OPO (OPOTEK, Carlsbad, CA, USA) used for this work has a pump wavelength of 1.064 µm (the fundamental wavelength of a Nd:YAG laser). The non-linear crystals are potassium titanyl phosphate (KTP) with a tuning range of range of 2.7-3.1 µm. The laser beam was focused onto the sample with a 250 or 50 mm focal length lens. The maximum laser repetition rate is 20 Hz with 5 ns pulse width corresponding to a maximum fluence of 30 kJ/m^2

and an irradiance of 500 MW/cm^2. The maximum laser energy is 2 mJ with no attenuation. The laser energy was attenuated using laser control software without compromising the energy stability. For attenuation, a polarizer mounted on a motorized rotation stage is automatically controlled by the software. In all experiments, the laser wavelength was set to 2.94 μm to overlap with the OH stretch absorption of water and analyte.[28] The 2.94 μm wavelength is also coincident with that of fixed wavelength Er:YAG lasers and selecting this wavelength on a tunable wavelength system is often done to facilitate comparison with the fixed wavelength laser.

2.5 Laser Ablation Sampling Collection Systems

Samples for ablation were deposited on a sample target and the sample target was placed below or held over a solvent (or surface). The distance between the target and the solvent (or surface) was typically less than 1 mm. The laser was focused onto the samples to ablate materials under ambient conditions. The ablated material was captured in the solvent (or on the surface) with different sampling systems. The captured materials were then analyzed using either the ion trap mass spectrometer or the MALDI mass spectrometer. Detailed information on each sampling system for capturing the laser ablated material is described in Chapters 3 - 6.

2.6 Reagents and Standards

The peptide and protein standards angiotensin II, neurotensin, bradykinin, leucine enkephalin, bovine insulin, equine cytochrome c, myoglobin, lysozyme, reagents trifluoroacetic acid (TFA), acetic acid, acetone and matrix compounds α-cyano-4-hydroxycinnamic acid (CCA), 2,5-dihydroxybenzoic acid (DHB), sinapinic acid (SA), and 4-hydroxy-3-methoxycinnamic acid (ferulic acid) were obtained from Sigma-Aldrich (St. Louis.MO, USA) and used without further

purification. Nitrocellulose was purchased from Schleicher & Schuell (Dassel, Germany). HPLC grade acetonitrile (ACN), and glycerol were purchased from Fisher Scientific (Pittsburgh, PA, USA) and house ultrapure water (18 MΩ cm, Barnstead E-pure System; Dubuque, IA, USA) was used. Analyte solutions for LAST LC-ESI/MS and LAST MALDI/MS were prepared by dissolving the peptide and protein standards in 1:1 (v/v) mixture of acetonitrile and 0.1% TFA to a concentration of 1 mM. Analyte solutions for LAST CE-ESI/MS were prepared by dissolving the standards in 1% acetic acid (pH = 2.8) solution for peptides and 0.1% acetic acid (pH = 3.3) for proteins to a final concentration of 1 mM. For the ablation transfer quantification studies, the peptide mixture solution was serially diluted in the 1% acetic acid solution from 100 µM to 1 µM. A saturated matrix solution for the peptide angiotensin II was prepared by dissolving 50 mg/mL of CCA in 1:1 (v/v) mixture of acetonitrile and 0.1% aqueous TFA and the solution for proteins and biological fluids (blood, milk, and egg white) was prepared by dissolving 35 mg/mL of ferulic acid in 1:1 (v/v) mixture of acetonitrile and 0.1% aqueous TFA. Chicken eggs and bovine milk were purchased from a local market. The egg whites were separated from the egg yolks for analysis.

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CHAPTER 3. INFRARED LASER ABLATION SAMPLE TRANSFER FOR MALDI AND ELECTROSPRAY*

This chapter describes work using an infrared laser to ablate materials under ambient conditions that were captured in solvent droplets. The droplets were either deposited on a target for off-line analysis by MALDI time-of-flight mass spectrometry or flow-injected into a nanoelectrospray source of an ion trap mass spectrometer. The infrared optical parametric oscillator (OPO) laser system at 2.94 μm wavelength and approximately 1 mJ pulse energy was focused onto samples for ablation at atmospheric pressure. The ablated material was captured in a solvent droplet 1-2 mm in diameter that was suspended from a silica capillary a few millimeters above the sample target. Once the sample was transferred to the droplet by ablation, the droplet was deposited on a MALDI target. A saturated matrix solution was added to the deposited sample, or in some cases, the suspended capture droplet contained the matrix. Peptide and protein standards were used to assess the effects of the number of IR laser ablation shots, sample to droplet distance, capture droplet size, droplet solvent, and laser pulse energy. Droplet collected samples were also injected into a nanoelectrospray source of the ion trap mass spectrometer with a 500 nL injection loop. It is estimated that pmol quantities of material was transferred to the droplet with an efficiency of approximately 1%. The direct analysis of biological fluids for off-line MALDI and electrospray was demonstrated with blood, milk, and egg. The implications of this IR ablation sample transfer approach for ambient imaging are discussed.

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3.1 Introduction

Lasers operating in the mid-IR wavelength region near 3000 nm wavelength have been used for MALDI,[2] but applications have been limited due to the large quantity of material ejected that is not compatible with commercial MALDI instruments operating at vacuum and with high extraction fields.[3,4] In contrast, the greater material removal of IR lasers is an advantage for ambient ionization techniques such as MALDESI and the related LAESI technique.[5-7] The volumetric energy deposition with a mid-IR laser is comparable to that of UV lasers, but the depth of laser penetration is an order of magnitude larger.[8] The greater penetration depth of IR lasers can be an advantage in removing deeply embedded materials such as biomolecules in polyacrylimide gels[9,10] and in tissue samples.[11]

For the work described in this chapter the mid-IR OPO laser system described in Chapter 2 was used to ablate biomolecules from samples under ambient conditions. The ejected material was captured in a solvent droplet that was held a few millimeters above the sample surface. The solvent droplet was then deposited on a MALDI target and analyzed in the Bruker Ultraflextreme MALDI TOF mass spectrometer or was flow-injected into the Hitachi ion trap MS. Angiotensin II was used to investigate the effects of the number of laser shots, distance between the droplet and target, droplet size, and droplet solvent. Proteins were tested with both MALDI and ESI analysis to investigate the ability of the IR laser to remove materials without fragmentation. Various real world samples were used to assess the ability of the IR laser to ablate biomolecules from complex mixtures. The implications of these results for ambient imaging of tissue samples with subsequent MALDI or ESI mass spectrometry are assessed.

3.2 Experimental

All experiments were carried out with the static sampling collection system. The static sampling collection system (Figure 3-1) consisted of a 3 mL syringe mounted on a manually controlled xyz stage (Model 461, Newport; Irvine, CA, USA) above a sample target that could be irradiated by the infrared laser. The syringe was connected to a Luer taper adaptor union that was connected to a 360 μm O.D. and 50 μm I.D. coated fused silica capillary. The capillary was 6 cm long and was held several millimeters above a stainless steel sample target. The syringe was operated manually and droplets of various sizes could be produced; a video camera and macro lens was used to observe the droplet.

The IR laser was used to ablate the sample from the metal target. The wavelength was set at 2.94 μm to overlap with the OH stretch absorption of the analyte and solvent[12] and the repetition rate was 2 Hz. The laser was directed at the target at a 45° angle and was focused onto the target with a 250 mm focal length lens. The maximum laser energy was 1.75 mJ with no attenuation. The pulse width is 5 ns corresponding to a maximum fluence of 3 J/cm^2 and an

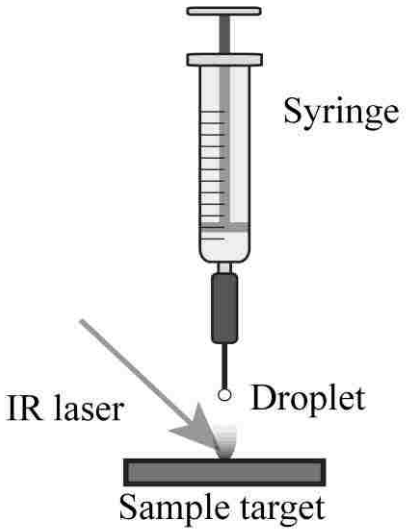

Figure 3-1. Schematic of the laser ablation sample transfer collection system.

irradiance of 500 MW/cm^2.

Samples for ablation were deposited on a stainless steel target and were irradiated while some solvent remained. The sample target was placed below the syringe and capillary and the distance between the target and droplet and the droplet diameter were manually adjusted. After the ablation of the sample and collection in the droplet, the syringe was removed from the xyz stage and the droplet was deposited on a MALDI target. For samples collected in a pure solvent, 2 μL of saturated matrix solution was added to the target; for samples collected in matrix solution, no additional matrix solution was added to the target. For electrospray analysis, the quadrupole ion trap mass spectrometer was used with a pulled nanospray tip. Tips were prepared by cutting a small length of 360 μm × 50 μm capillary with a binder clip suspended at the bottom end and heating the middle with a flame.[13] No nebulizing gas was used for the electrospray. After laser ablation sample transfer to the liquid droplet, the droplet solution was delivered to a 500 nL injection loop (Model 7410; Rheodyne, Cotati, CA, USA) connected to the nanoelectrospray source. MALDI mass spectra were obtained on the Bruker MALDI-TOF mass spectrometer or, for electrospray analysis, the Hitachi M8000 ion trap mass spectrometer was used.

Analyte solutions were prepared by dissolving angiotensin II, bovine insulin and cytochrome c in a 1:1 (v/v) mixture of acetonitrile and 0.1% TFA to a concentration of 1 mM. A saturated matrix solution for the peptide angiotensin II was prepared by dissolving 50 mg/mL of CCA in 1:1 (v/v) mixture of acetonitrile and 0.1% aqueous TFA and the solution for proteins and biological fluids (blood, milk, and egg white) was prepared by dissolving 35 mg/mL of ferulic acid in 1:1 (v/v) mixture of acetonitrile and 0.1% aqueous TFA. Ten μL of the egg white or the bovine milk were deposited onto a stainless steel target for collecting materials without any

sample pretreatment. Undiluted human blood (10 μL) was obtained from a volunteer and transferred to a stainless steel target with a micropipette.

3.3 Results

An important issue in laser ablation sample transfer to solvent droplets or other collection devices is whether biomolecules can be transferred without fragmentation. Another issue is the efficiency of sample transfer: whether a sufficient quantity of material can be transferred to achieve a mass spectrometry or other analysis. Our initial studies involved the use of mid-infrared laser ablation to transfer pure peptides and proteins for MALDI analysis to assess the ability to transfer these molecules without fragmentation using mid-IR wavelengths. Next, the ability of the mid-IR laser to manipulate complex biological samples was assessed. These studies were then extended to electrospray mass spectrometry for IR laser ablation droplet capture and nanospray flow-injection analysis.

3.3.1 IR Laser Ablation Sample Transfer for MALDI

MALDI mass spectra of the peptide angiotensin II prepared by pulsed infrared laser ablation droplet capture are shown in Figure 3-2. A 10 μL volume of a 1 mM solution of angiotensin II in a 1:1 (v/v) solution of ACN and 0.1% TFA was deposited on a stainless steel target that was held 3 mm below a solvent droplet. The solvent in the droplet was also a 1:1 (v/v) solution of ACN and 0.1% TFA and was approximately 1.5 mm in diameter. Material was ablated from the target at 2.94 μm and 1 mJ energy and captured in the solvent droplet then deposited on a standard MALDI target. The 2.94 μm wavelength was chosen because it has been used most often in mid-IR ablation[2] and also because this wavelength is near the peak of the OH

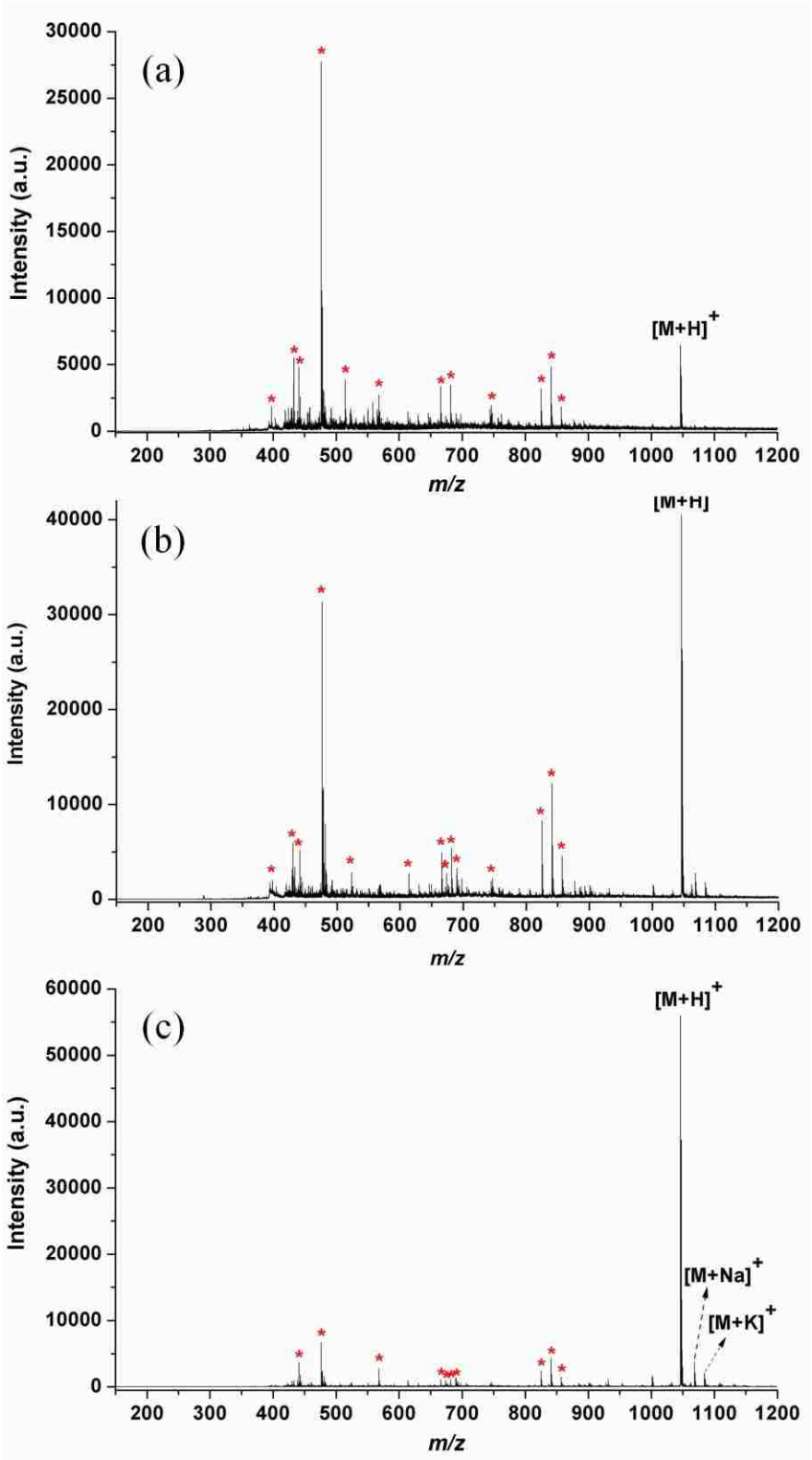

Figure 3-2. MALDI mass spectra of the peptide angiotensin II with sample delivery by ambient infrared laser ablation sample transfer using different numbers of laser shots: (a) 60 laser shots, (b) 180 laser shots, (c) 300 laser shots. Matrix peaks are indicated with asterisks.

stretch absorption of water and other solvents and the strong absorption leads to the vigorous evolution of plume material and the production of large quantities of particulate.[14,15] A 2 μL volume of saturated CCA matrix in a 1:1 (v/v) solution of ACN and 0.1% TFA was deposited with the droplet. MALDI mass spectra were obtained in reflectron mode and were the sum of 500 UV laser shots. Figure 3-2a was obtained from 60 infrared laser ablation shots, Figure 3-2b from 180 laser ablation shots, and Figure 3-2c from 300 laser ablation shots. In Figure 3-2a, the large peaks (indicated with asterisks) in the low mass region of the mass spectrum correspond to matrix, matrix cluster, and matrix fragment ions typical for this matrix.[16-18] At 180 IR laser shots, the protonated angiotensin II ion is the most intense peak and, at 300 shots, the protonated molecule peak is the most intense peak with only a few other less intense peaks in the mass spectrum.

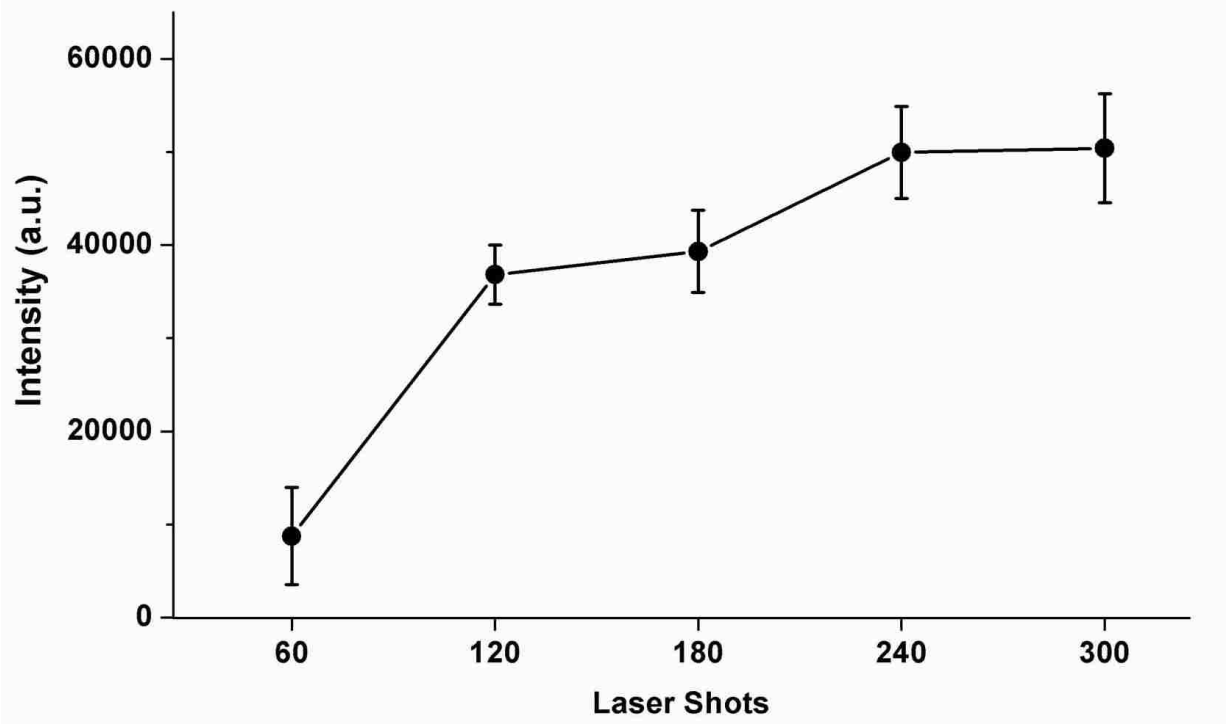

Figure 3-3. MALDI signal intensity for angiotensin II prepared by IR laser ablation plotted as a function of the number of IR laser shots for sample transfer.

Optimization of the ablation setup included the number of laser shots, distance between the droplet and sample, droplet diameter, solvent, and laser energy. A plot of the signal intensity (peak height) as a function of the number of laser shots show that the signal increases rapidly between 60 and 120 laser shots and levels off somewhat after several hundred laser shots (Figure 3-3). This is likely due to sample removal by ablation at the 1 mJ laser energy. The distance between the droplet and the target was varied from 1 to 5 mm, and it was found that the closest distance gave the greatest signal intensity (Figure 3-4). This is consistent with the relatively large radial divergence observed in IR laser ablation plumes.[15,19,20] Additionally, the stopping distance for a plume of ablated material at atmospheric pressure is several millimeters, and target to

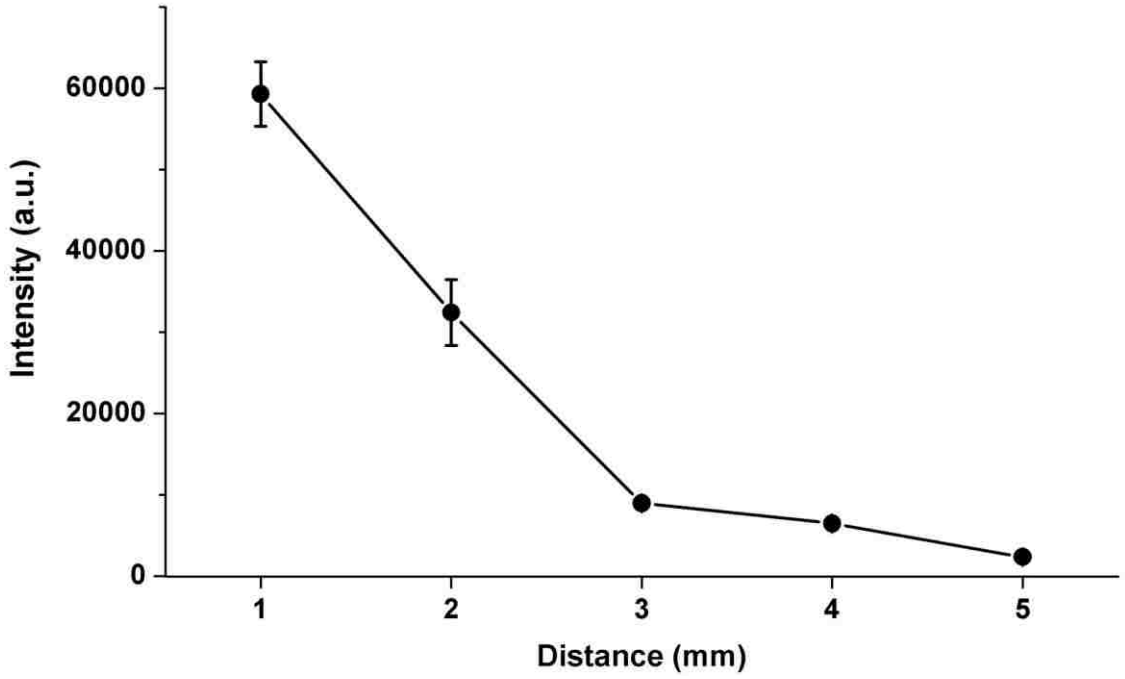

Figure 3-4. MALDI signal intensity for angiotensin II prepared by IR laser ablation plotted as a function of the distance between the sample target and bottom of the collection droplet.

droplet distances larger than this may prevent particles from reaching the droplet. A relatively large droplet size is favored even though this leads to a dilution of the sample in a greater solvent volume. The signal is greatest for the largest droplet size (2 mm diameter; Figure 3-5). The

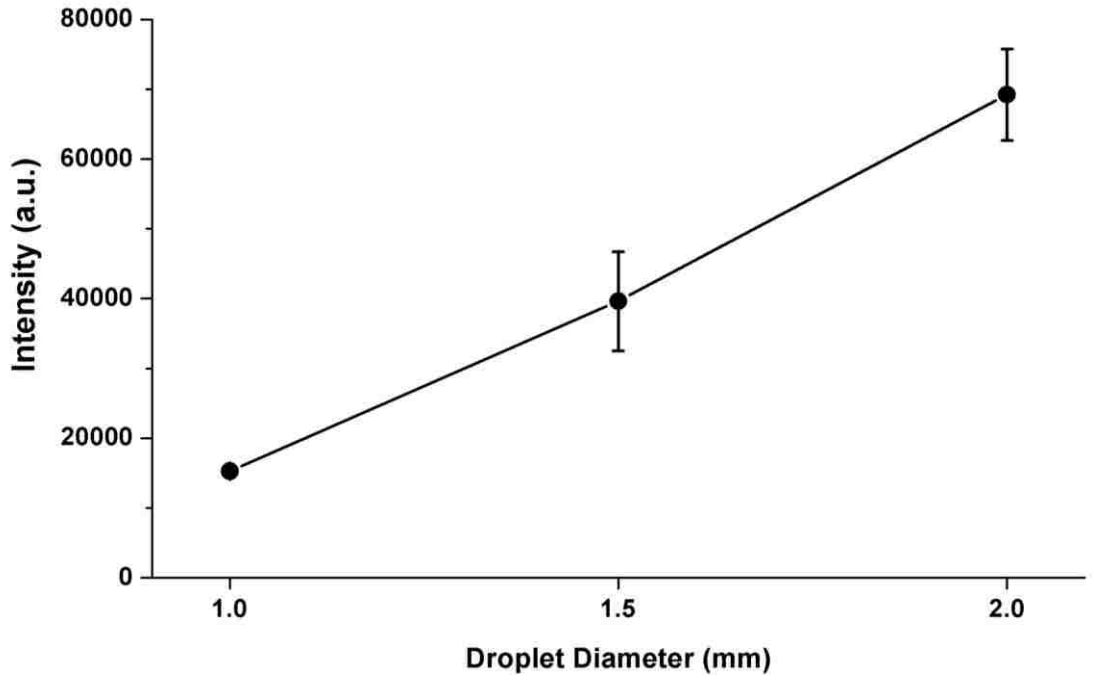

Figure 3-5. MALDI signal intensity for angiotensin II prepared by IR laser ablation plotted as a function of the diameter of the capture droplet.

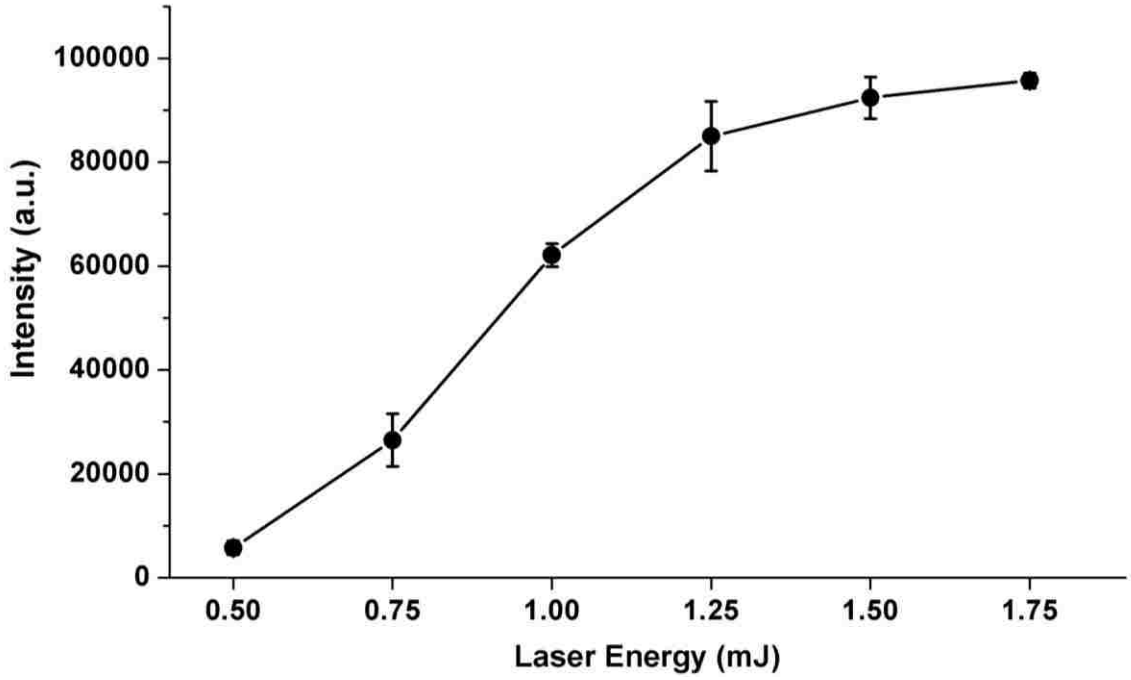

Figure 3-6. MALDI signal intensity for angiotensin II prepared by IR laser ablation plotted as a function of the infrared laser pulse energy.

signal increased by a factor of five when the droplet volume increased by eight times, suggesting that the larger droplet is captures more material from the expanding plume. The laser energy was varied from 0.5 to 1.75 mJ per pulse and higher pulse energies give the greatest signal, but it does not increase as rapidly above 1 mJ (Figure 3-6). This effect has been observed previously and is attributed to shielding of the target by the dense plume formed at higher energies.[14] No additional peptide fragmentation was observed at the higher pulse energies.

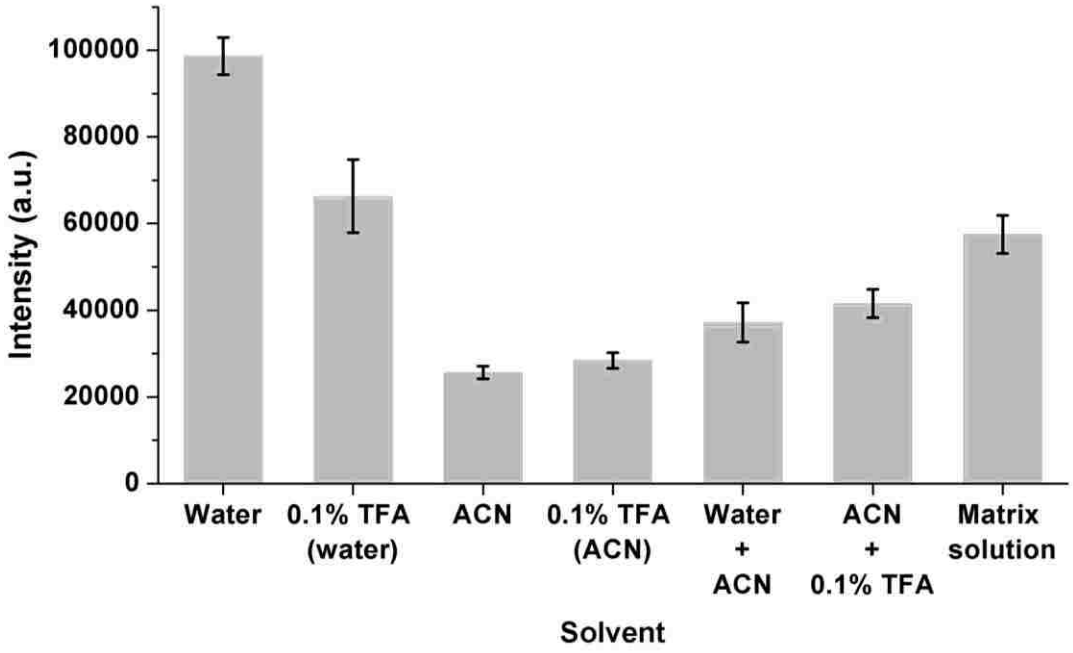

Figure 3-7. MALDI signal intensity for angiotensin II prepared by IR laser ablation using different droplet capture solvents.

Different solvents were tested in the capture droplet: water, 0.1% TFA in water, ACN, 0.1 % TFA in acetonitrile, 1:1 (v/v) ACN and water, and 1:1 (v/v) ACN and 0.1% TFA in water (Figure 3-7). The best solvent is pure water, which is most likely due to the higher solubility of the peptide in this solvent. The drawback to the pure water solvent is the longer time that is required for solvent evaporation when the droplet is deposited on the MALDI target. An ACN and 0.1% TFA solvent that also contained the matrix was found to produce mass spectra

comparable to those where the matrix was added separately. A difficulty with the matrix in capture droplet approach is that matrix crystals tend to form in the capture droplet as the solvent evaporates.

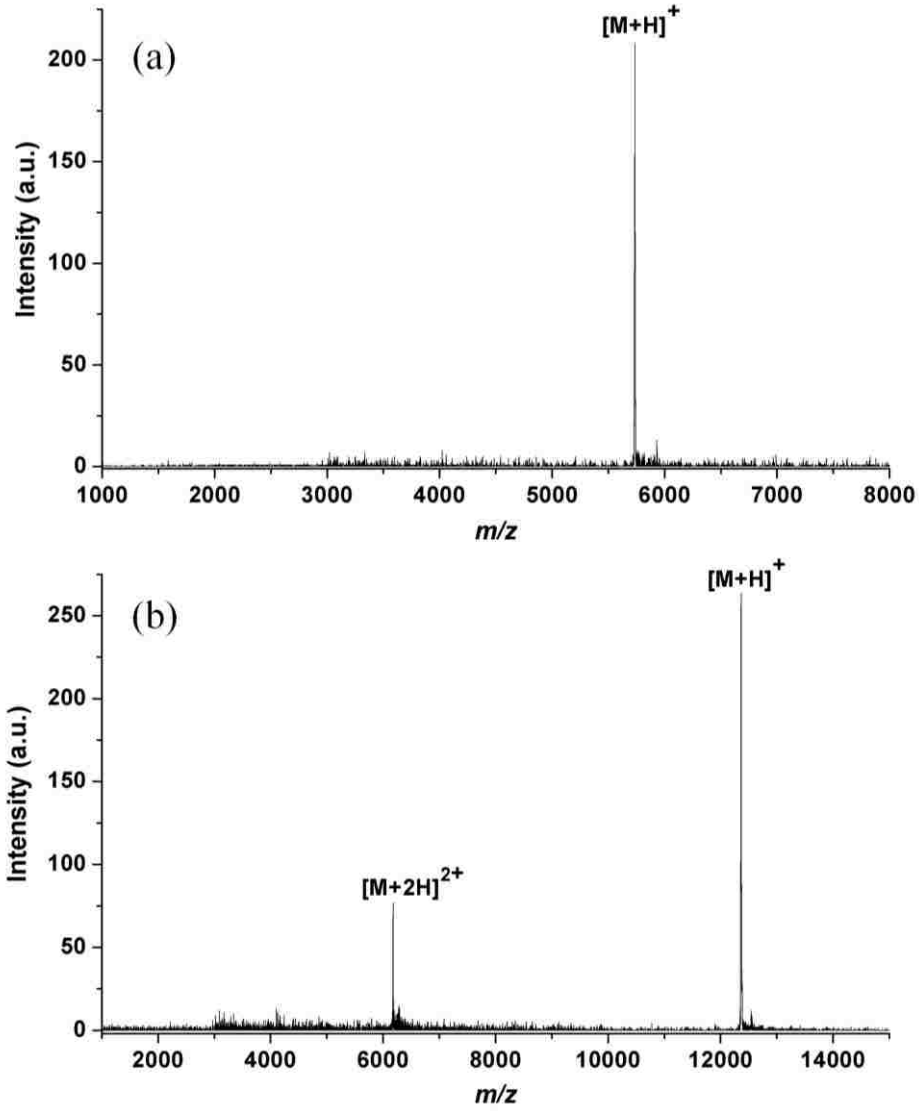

Figure 3-8. Infrared laser ablation sample transfer MALDI mass spectra of (a) bovine insulin and (b) cytochrome c.

It was found that proteins could also be transferred to the droplet by IR laser ablation without fragmentation. Mass spectra of bovine insulin and cytochrome c are shown in Figure 3-8. The proteins were deposited on the ablation target from 1 mM solutions in 1:1 (v/v) ACN and

0.1% TFA and 1.5 mJ laser energy to transfer the material from the target to the droplet. Ferulic acid matrix was added with the laser ablation capture droplet to the MALDI target. Other conditions were a 2 mm capture droplet, 1 mm target to droplet spacing, and 60 laser shot collection.

Several samples were used to test to the ability of laser ablation to analyze complex mixtures of biological molecules; mass spectra are shown in Figure 3-9. In all cases, a total of 60 laser shots at 1.5 mJ energy was used to transfer the material from the target into a 2 mm diameter droplet suspended 1 mm above the target. A ferulic acid matrix was used. A mass spectrum from an analysis of whole human blood is shown in Figure 3-9a. A droplet of blood from a volunteer was deposited on a metal target and irradiated with the IR laser and the laser-transferred material was analyzed by UV MALDI. Two sets of peaks corresponding to the hemoglobin α- and β-chains without heme were observed and are indicated in Figure 3-9a. A signal corresponding to heme that was more than 30 times stronger than the α- and β -chain peaks was observed at *m/z* 617 (not shown). Heme loss and α- and β-chains fragmentation is typically observed in MALDI analysis of whole blood.[21] A MALDI mass spectrum of undiluted whole blood showed a broad unresolved feature between 500 and 4000 *m/z* (Figure 3-10a). A mass spectrum obtained from whole milk is shown in Figure 3-9b. Several proteins were observed: proteopeptone pp81, γ_3-casein/ γ_2-casein/ γ_1-casein, α-lactalbumin, β-lactalbumin B and A, and α_{S1}-casein/β-casein. The assignment of the peaks is based on previously reported spectra reported for MALDI analysis of milk,[22,23] the spectrum in Figure 3-9b is similar to that reported previously for diluted milk. A MALDI mass spectrum of a direct deposit of milk was dominated by the α-lactalbumin peak (Figure 3-10b), suggesting that there may be signal suppression effect in the direct MALDI analysis that is avoided by the laser ablation and droplet

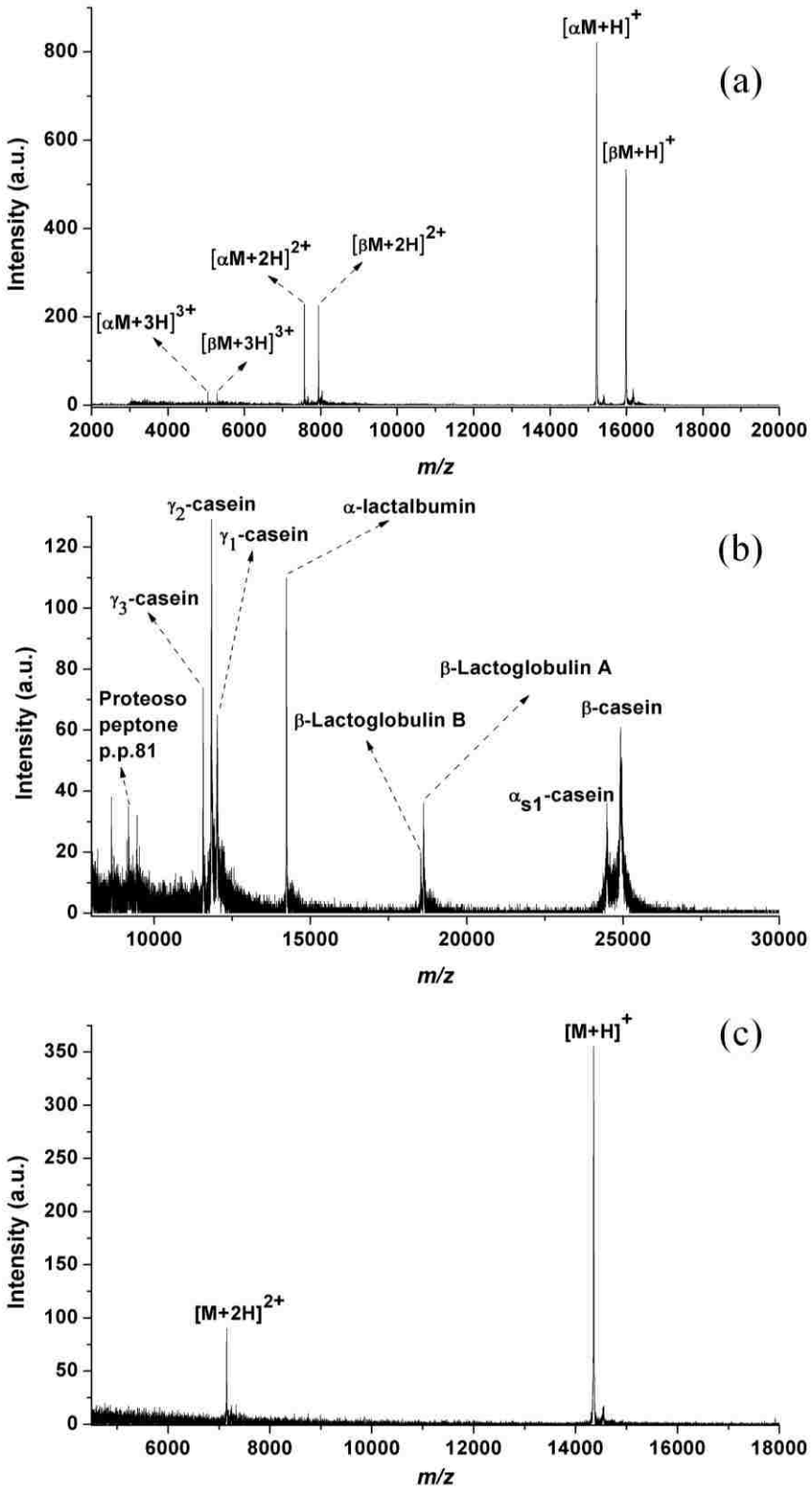

Figure 3-9. Infrared laser ablation sample transfer MALDI mass spectra of (a) human blood, (b) whole milk, and (c) egg white.

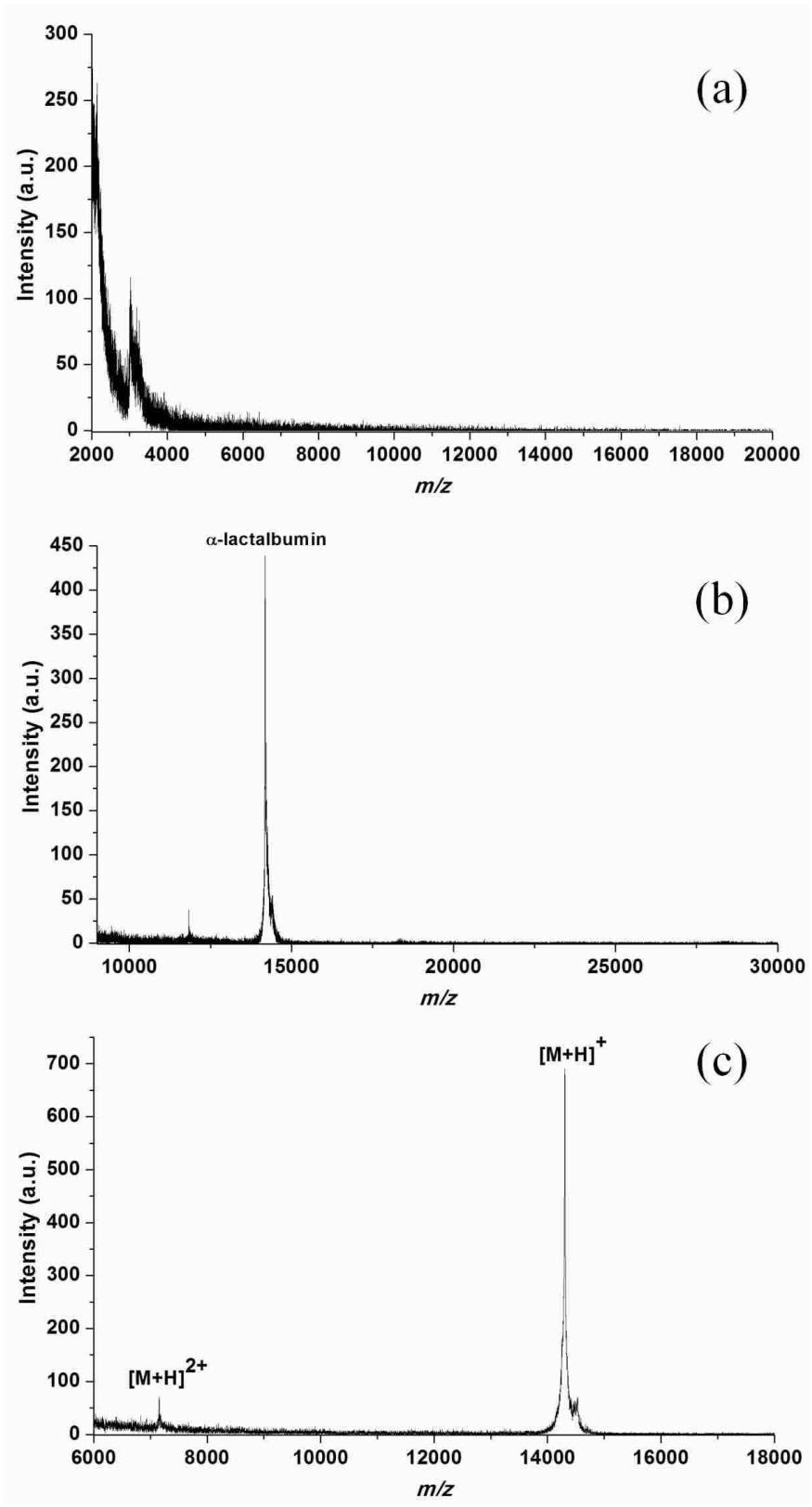

Figure 3-10. Direct MALDI mass spectra of (a) human blood, (b) whole milk, and (c) egg white.

capture approach. Egg white from a fresh egg with laser ablation sample treatment led to the mass spectrum shown in Figure 3-9c. Both singly and doubly protonated lysozyme was observed with the latter significantly less intense. The mass spectrum is similar to direct MALDI of egg white (Figure 3-10c).

3.3.2 IR Laser Ablation Sample Transfer for Electrospray

Electrospray mass spectrometry was accomplished with samples that were infrared laser ablated from a stainless steel target at 2.94 μm wavelength and captured in a 2 mm droplet held 1 mm above the target. Mass spectra of pure peptides and proteins are shown in Figure 3-11. A nanoelectrospray mass spectrum of angiotensin II prepared by infrared laser ablation sample transfer is shown in Figure 3-11a. A 10 μL volume of a 1 mM solution of angiotensin II in 7:3 (v/v) ACN and 0.1% TFA was deposited on the target. The sample was ablated by 300 infrared laser ablation shots at 2.94 μm and 1.5 mJ energy. The droplet was manually withdrawn into a 10 μL syringe and delivered to a 500 nL injection loop for delivery to the electrospray. The peptide signal is half as intense as a 500 nL flow injection of a 10 μM solution of peptide, suggesting that the concentration of the peptide in the droplet approximately 5 μM. For a 2 mm droplet, this corresponds to 40 pmol of peptide transferred to the droplet. Under similar IR laser ablation conditions, it was found that approximately 4000 glycerol particles averaging 1 μm in diameter are ablated from a metal target per laser shot, and that these particles accounted for a significant fraction of the ablated material.[24] If a similar quantity of solid peptide is ablated in the droplet capture experiments, the transfer of 40 pmol to the droplet would require a 1% capture efficiency.

Nanoelectrospray mass spectra of proteins transferred from target to droplet by IR laser ablation are shown in Figure 3-11b (bovine insulin) and Figure 3-11c (cytochrome c). The capture conditions were identical to those for the peptide. The mass spectrum of bovine insulin

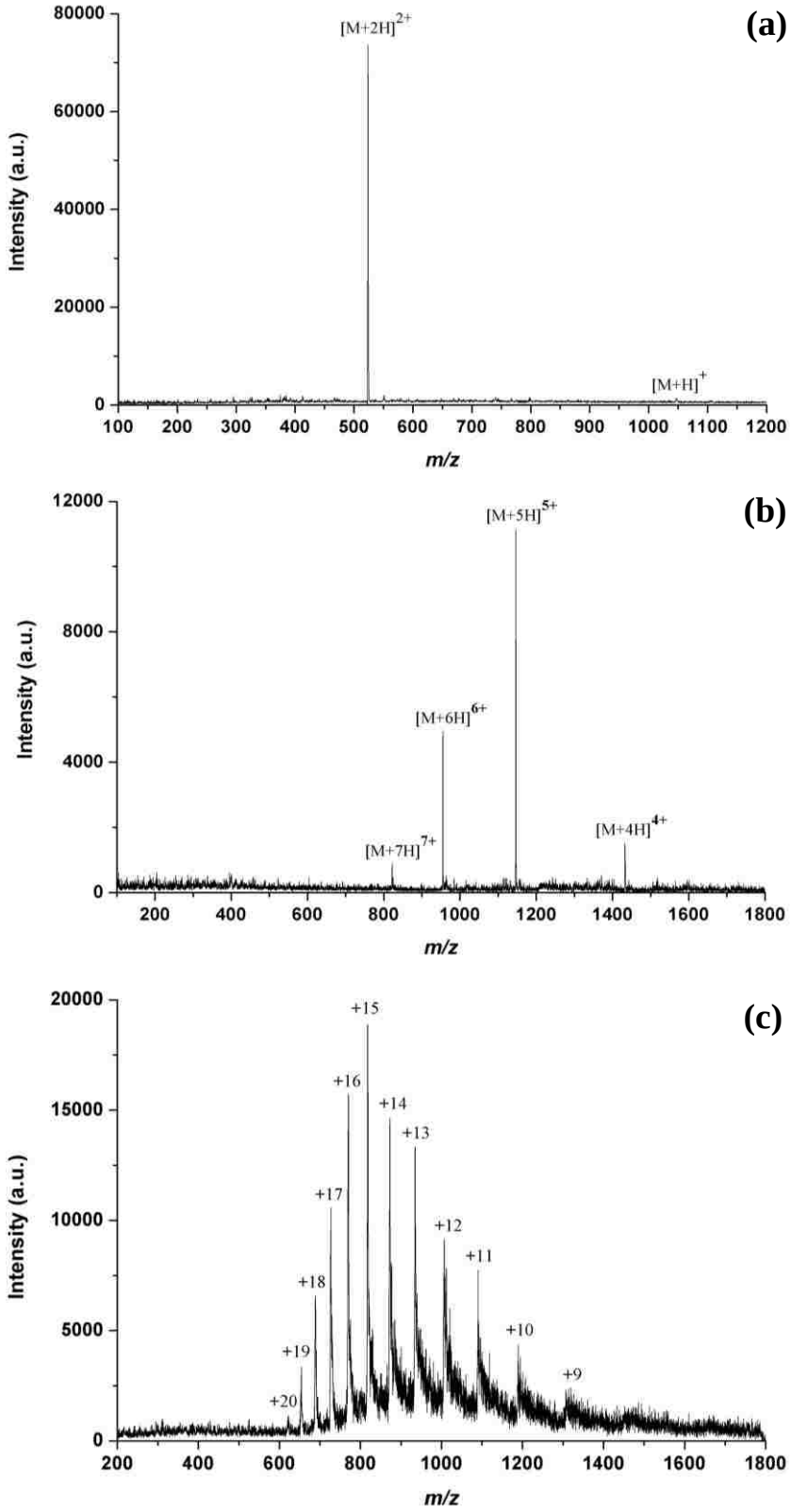

Figure 3-11. Nanoelectrospray mass spectra of (a) angiotensin II (b) bovine insulin, and (c) cytochrome c prepared by infrared laser ablation sample transfer.

shows multiply charged ions from $[M + 7 H]^{7+}$ to $[M + 4 H]^{4+}$ and multiply charged cytochrome c ions from $[M + 20 H]^{20+}$ to $[M + 9 H]^{9+}$ are observed. No dimers or trimers are observed in both cases. The mass spectrum from each droplet was similar to those obtained from the direct injection of a 10 μM solutions of bovine insulin and cytochrome c into a 500 nL injection loop (data not shown). The similarity of the apparent transfer efficiency suggests that there is not a significant mass discrimination effect in the laser ablation transfer efficiency.

Several samples were used to test infrared laser ablation sample transfer for the analysis of complex biological mixtures. Mass spectra of various biological fluids are shown in Figure 3-12. In all cases, a total of 300 laser shots at 2.94 μm wavelength and 1.5 mJ laser energy was used to transfer the sample materials from a metal target into a 2 mm diameter droplet suspended 1 mm above the metal target. A mass spectrum from whole human blood obtained using IR laser ablation sample transfer is shown in Figure 3-12a. A droplet of blood from a volunteer was deposited on a metal target and irradiated with the IR laser. The laser-transferred material was ionized with a nanoelectrospray source. In the mass spectrum, two sets of peaks corresponding to distributions of multiply-charged ions can be observed that correspond to the hemoglobin α- and β-chains without the heme group and charge states $[M + 12 H]^{12+}$ to $[M + 23 H]^{23+}$. The peak at *m/z* 616 corresponds to the free heme. Several other singly and multiply charged ions are also observed. On the basis of the published data[6,25-28] and the Human Metabolome Database,[29] the peaks at *m/z* 1125, 1069, and 1093 may result from α heme (+14), glycated α- (+15) and β-chains (+14), respectively. The peaks indicated with asterisks may originate from phosphocholine (PC); the peaks at *m/z* 413, 742, and 784 may correspond to protonated LysoPC (10:0), PC (15:0/18:3), and PC (14:1/22:2), respectively.

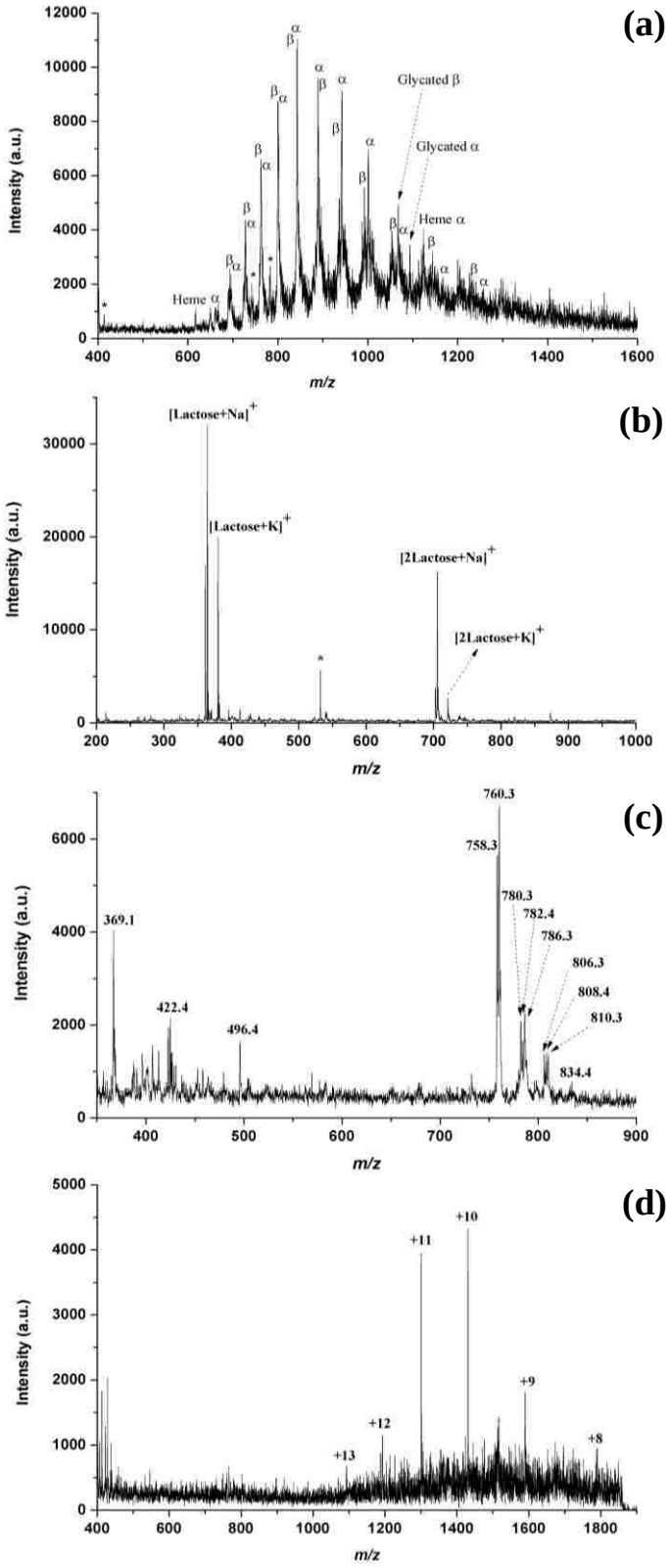

Figure 3-12. Nanoelectrospray mass spectra of (a) human blood, (b) whole milk, (c) egg yolk, and (d) egg white prepared by infrared laser ablation sample transfer.

A mass spectrum obtained from whole milk is shown in Figure 3-12b. The abundant peaks correspond to the sodium and potassium adducts of lactose as well as the sodium and potassium adducts of the dimers of lactose. The assignment of the peaks is based on previously reported mass spectra.[30-32] The additional ion at *m/z* 533 (indicated with an asterisk) corresponds to a singly charged lipid ion or other component of the milk. The mass spectra of chicken egg yolk and egg white from a fresh egg are shown in Figure 3-12c and d, respectively. Figure 3-12c shows several peaks from phosphatidylcholine (PC) with different fatty acid compositions; PC 16:0/18:2 (*m/z* 758), PC 16:0/18:1(*m/z* 760), PC 16:0/20:4 (*m/z* 782), PC 18:0/18:2 (*m/z* 786), PC 18:0/20:4 (*m/z* 810), PC 18:0/22:6 (*m/z* 834). Sodium adducts phosphatidylcholine ions are also observed at *m/z* 780 and 808. The peak at *m/z* 496 is attributed to lysophosphatidyl-choline containing one palmitic acid substitution. The peaks at *m/z* 422 and 369 are attributed to 1,2-dilaurylpho-sphatidylcholine by loss of an acyl group and from cholesterol by the loss of water.[5,25,33-35] A mass spectrum obtained from egg white is shown in Figure 3-12d. Multiply-charged ions from lysozyme[36] ($[M + 13H]^{13+}$ to $[M + 8H]^{8+}$) and unidentified ions (<*m/z* 500) are observed

3.4 Summary

Laser ablation sample transfer was demonstrated for ambient sampling and subsequent MALDI analysis using a commercial TOF mass spectrometer and ESI analysis using an ion trap mass spectrometer. With the use of a mid-infrared OPO operating in the 2.94 μm wavelength region, it was possible to ablate material from solid samples, capture the material in a solvent droplet, and create ions both by conventional MALDI and conventional electrospray approaches. Due to the nature of the expanding plume of material, it was found that the optimum conditions

for sampling are a relatively large 2 mm solvent droplet held close to the sample (1 mm) so as to capture the largest quantity of sample. The efficiency of material capture in the droplet is estimated to be 1% with approximately 10 pmol estimated to be transferred to the droplet in a typical experiment. The best solvent for plume capture for MALDI is water, although an equal volume mixture of 0.1% TFA in water and acetonitrile resulted in faster evaporation when the droplet was placed on a MALDI target. More than 1 mJ IR laser energy could be used for laser ablation sample transfer without sample degradation observed for either peptides or proteins observed with either electrospray or MALDI. The laser fluence was 1000 times greater than that used for UV ablation/electrospray[37] and 10 times greater than for near IR ablation/MALDI[38] yet little to no fragmentation of biomolecules was observed. The infrared laser ablation sample transfer was used to analyze complex biological mixtures for MALDI and ESI analysis.

The mechanism of sample transfer is either through the ejection of free molecules or particulate. Previous studies suggest that a significant fraction of the material ejected by the IR laser is ablated as coarse particulate.[15,24] Additional material is ejected as smaller particles and free molecules. All of these ejected materials could be captured by the suspended droplet and contribute to the resulting MALDI or ESI signal. Microscopy studies of material collected as thin films on solid targets could help to quantify the ratio of free to particulate material ejected from samples by laser ablation.[39]

Laser ablation sample transfer opens up some new possibilities for ambient imaging both with MALDI and electrospray ionization. Laser desorption-based imaging mass spectrometry is currently done under vacuum using MALDI directly from tissue to which matrix has been added.[40] Drawbacks to this approach are the need to add matrix directly to the tissue and the requirement for analysis with the sample under vacuum. Ambient imaging using laser desorption

coupled with electrospray ionization is currently under development and infrared lasers show great promise due to their ability to efficiently remove material.[11] Laser ablation sample transfer decouples the ablation of material from the ionization and the efficiency of material transfer is high. One possible adaptation of IR laser ablation transfer is to ablate material from a tissue sample for direct collection on a MALDI target. Scanning the IR ablation laser across the tissue will ablate biomolecules that are collected on a matrix coated or nanostructured matrix-free target. Transmission geometry IR laser ablation would allow the separation between the tissue and MALDI target to be reduced to less than a mm spacing and allow tight focusing of the ablation laser beam.

Coupling spatially resolved IR laser ablation of tissue with electrospray can be done either off-line or on-line. In the off-line mode, the sample is ablated into droplets that are delivered sequentially to the electrospray source. With the on-line approach, the laser scans across the tissue sample and ablates material into an exposed flowing stream of solvent that carries the ablated material to the electrospray source. The basic principle of this approach has been described[41] and demonstrated with UV laser ablation.[37]

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CHAPTER 4. INFRARED LASER ABLATION SAMPLE TRANSFER FOR ON-LINE LIQUID CHROMATOGRAPHY ELECTROSPRAY IONIZATION MASS SPECTROMETRY*

In this chapter, an on-line laser ablation sampling system and coupling of the system to liquid chromatography (LC) using an infrared (IR) laser to ablate and transfer materials into a flowing solvent stream is described. With this approach, samples were deposited on a microscope slide mounted on a translation stage and ablated in transmission geometry using the mid-IR OPO system. The ablated material was captured in an exposed flowing solvent stream that carried the ablated material to the electrospray source of the ion trap mass spectrometer. Post-ablation separation was accomplished using a capillary column downstream of the capture zone. The performance of the system was assessed using peptide and protein mixtures ablated from the target and analyzed with and without LC separation.

4.1 Introduction

Ambient ionization has been coupled with various separation methods, including liquid chromatography (LC), which allows the direct analysis of separation media or effluent without further processing (see Chapter 1).[2-12] However, this approach still requires sample collection steps before loading samples onto the LC.

The goal of the work described in this chapter was to demonstrate ambient sampling coupled with a LC separation before ionization as an initial step toward coupling laser ablation imaging with liquid separations. This work builds on the static capture electrospray described in Chapter 3. The pulsed mid-IR laser was tuned to the OH stretch absorption that efficiently

* The work reported in this chapter has been published in the *Journal of Mass Spectrometry*.[2] Reprinted by permission of the John Wiley and Sons.

ablates large biomolecules with little fragmentation.[13] In this work, on-line laser ablation sample transfer was used with a flowing solvent stream and the coupling of this system to LC was demonstrated.

4.2 Experimental

The mid-IR laser ablation setup used for on-line ESI is based on the system described in Chapter 2 and the static system described in Chapter 3. Samples were deposited on an indium tin oxide coated quartz microscope slide (Bruker) mounted on a three-axis translation stage (M-433, Newport, Irvine, California) operated using actuators (LTA-HS, Newport) and motion controller (ESP300, Newport) as indicated in Figure 4-1. The slide was held over an exposed flowing solvent stream and irradiated in transmission geometry with the wavelength tunable pulsed IR optical parametric oscillator. The laser beam was directed at the sample target at a 90° angle and focused onto the sample with a 50 mm focal length lens. The laser pulse width was 5 ns, the repetition rate was 20 Hz and the wavelength was set at 2.94 μm to overlap with the OH stretch absorption of the analyte and solvent.[13,14] The maximum laser energy was 2.0 mJ, corresponding to a fluence of 30 kJ/m^2 at the target.

The open solvent stream was constructed using two lengths of 360 mm O.D. × 50 mm I.D. silica capillary and 2 cm of 1.6 mm O.D. PEEK tubing sheath. A 1 mm section of the PEEK tubing was removed to expose the channel, and the two silica capillaries were inserted into the sheath with a 1 mm spacing between them at the gap. One of the silica capillaries was connected to a syringe pump and the other to the ESI source. The commercial electrospray tip was surrounded by a stainless steel tube that was 800 mm ID and 1.5 mm OD with a 400 mm ID tapered tip through which nebulizing gas was provided to create a pneumatically assisted

electrospray. The flow of solvent to the capture zone was balanced by the removal of solvent by the local vacuum generated by the pneumatic nebulization.[15] The point of laser ablation on the microscope slide was held at a distance of 1 mm above the capture zone.

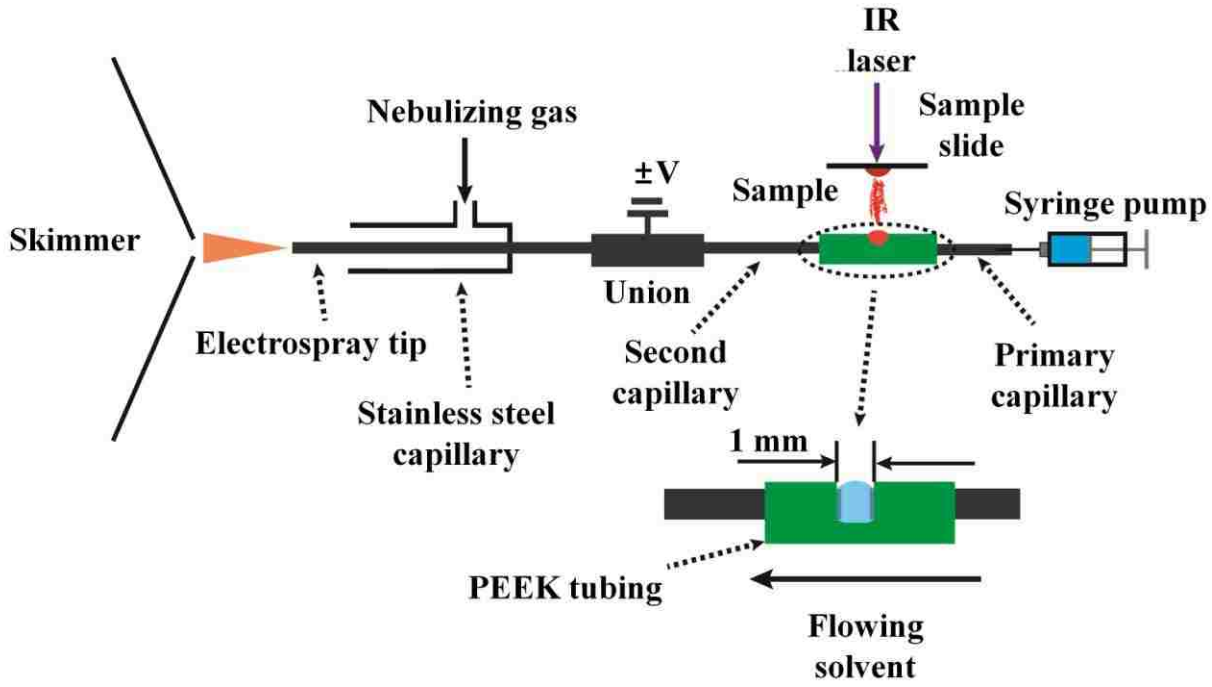

Figure 4-1. Schematic of the laser ablation sample transfer collection system for on-line ESI.

To perform LC, a 300 mm I.D. × 15 cm C18 capillary column (Acclaim PepMap300, Dionex, Sunnyvale, CA), LC pump (Model 196-31; Milton Roy Co., St. Petersburg, FL) and injection valve (Model 7010; Rheodyne, CA, USA) with a 5 mL sample loop were added to the system as shown in Figure 4-2. With the injection valve in the load position (Figure 4-2a), flow from the capture zone flowed to waste through the sample loop driven by a peristaltic pump (Model 3386; Control Company, Friendswood, Texas), while the in acetonitrile and 0.1% aqueous trifluoroacetic acid (TFA) (70:30, v/v) solvent from the LC pump flowed to the ESI source through the LC column. After a 20 s laser ablation sampling time to allow the captured

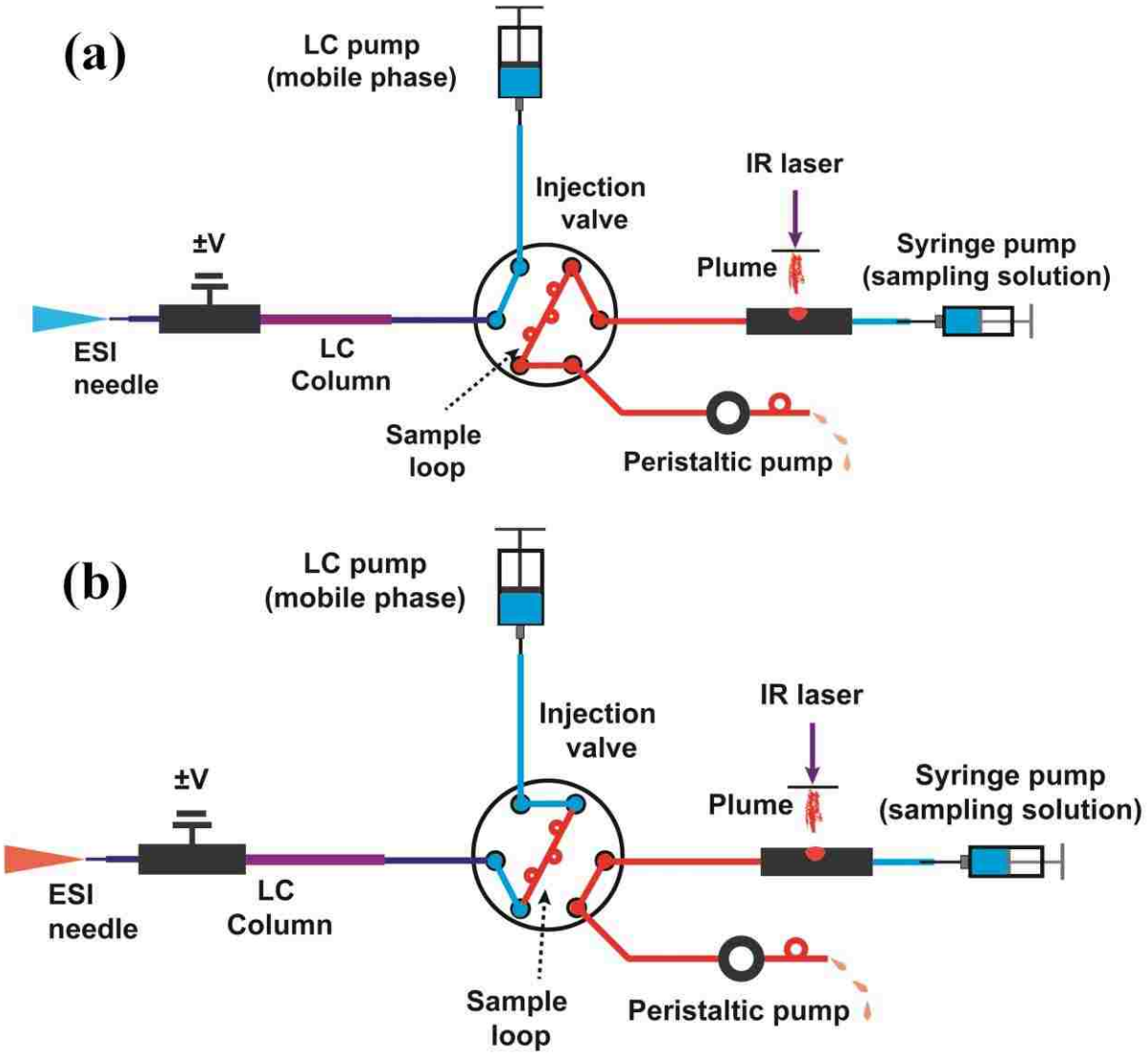

Figure 4-2. Schematic of the laser ablation sample transfer system for coupling with liquid chromatography: a) sample loading and b) injection.

material to flow into the sample loop, the valve was switched to the inject position (Figure 4-2b). The solution containing ablated material in the sample loop was delivered to the ESI source through the LC column, and the transit time from the collection point to the electrospray sources was approximately 15 s. A typical separation under these conditions required between 5 and 30 min.

Mass spectra and total ion current (TIC) chromatograms were acquired using the ion trap mass spectrometer. Mass spectra were acquired at two microscans in positive ion mode, and ions were accumulated for 100 ms in the trap. The skimmer cone was held at 3.5 kV and heated to a temperature of 180 ^{o}C.

The peptide and protein standards angiotensin II, bradykinin, leucine enkephalin, bovine insulin, equine cytochrome c, myoglobin, lysozyme and reagent TFA were used without further purification. HPLC grade acetonitrile and house ultrapure water was used. Analyte solutions were prepared by dissolving the peptide and protein standards in 1:1 (v/v) mixture of acetonitrile and 0.1% TFA to a concentration of 1 mM.

4.3 Results

Initial tests were performed with peptide standards deposited on the sample slide for ablation into the flowing solvent. A 1 mM solution of angiotensin II in 1:1 (v/v) acetonitrile and 0.1 % aqueous TFA was used to prepare five sample spots using a 10 mL volume for each deposited on a microscope slide. The slide was then mounted on the translation stage above the solvent capture zone. The flow of solvent was adjusted to match the electrospray rate at approximately 2 mL/min. Each sample spot was irradiated in turn by the IR laser with a different number of laser shots (100, 150 and 300) and different laser energies (0.8, 0.5, and 0.3 mJ). Figure 4-3 shows the TIC for the five sample spots: 1) 300 laser shots at 0.8 mJ, 2) 150 laser shots at 0.8 mJ, 3) 100 laser shots at 0.8 mJ, 4) 100 laser shots at 0.3 mJ and 5) 100 laser shots at 0.5 mJ. After ablating each spot, the laser was turned off and the slide held in place for 60 s and then moved to the next spot. Moving from spot to spot took approximately 30 s. The TIC signal

persists for approximately 30 s after the laser is turned off, indicating a memory effect due to capture zone dead volume or slow dissolving and mixing of the ablated material. The size of the

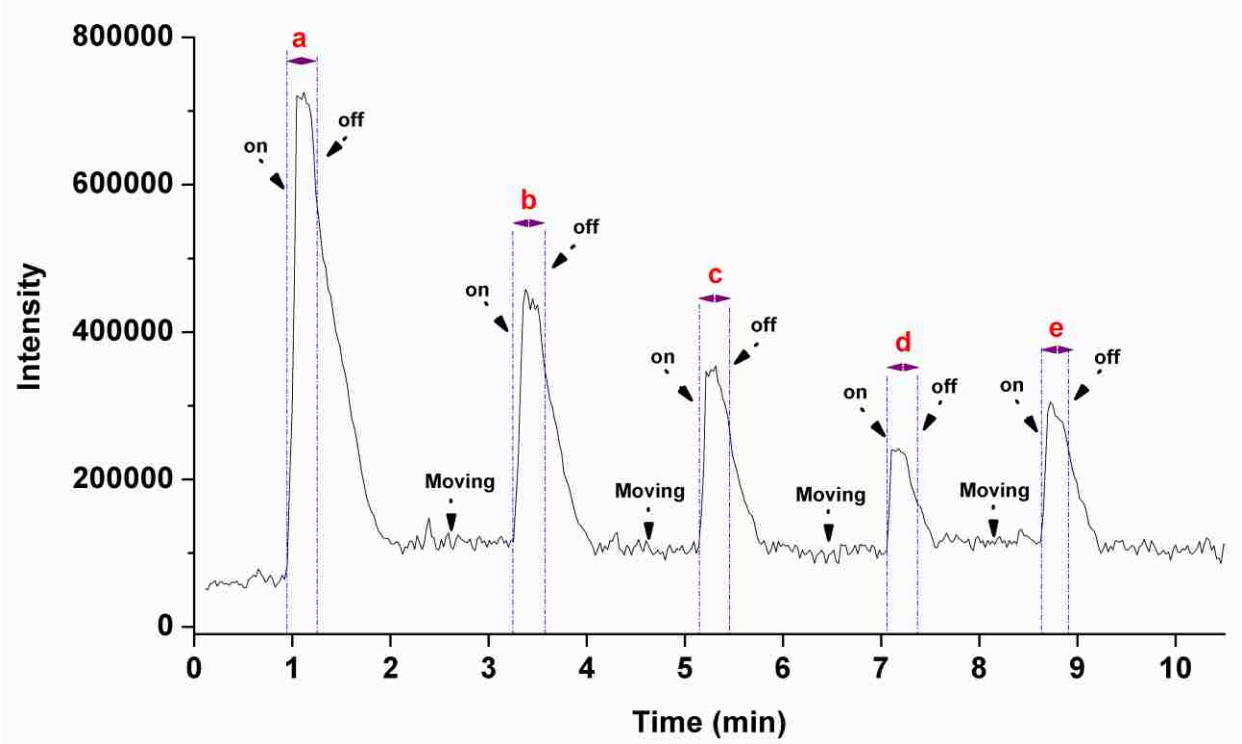

Figure 4-3. Total ion current (TIC) from laser ablation of the five angiotensin II spots under different laser conditions: a) 300 laser shots at 0.8 mJ, b) 150 laser shots at 0.8 mJ, c) 100 laser shots at 0.8 mJ, d) 100 laser shots at 0.3 mJ and e) 100 laser shots at 0.5 mJ. Arrows indicate the time at which the sample slide was moved to the next sample spot.

ablation plume is much larger than the capture zone;[16] therefore, there may be a significant quantity of ablated material transferred to the region surrounding the solvent at the capture zone. This material could enter the solvent stream more slowly than that directly captured by the solvent.

Figure 4-4 shows representative mass spectra from the peptide ablation experiment depicted in Figure 4-3. Figure 4-4a is the averaged mass spectrum corresponding to a) in Figure 4-3 (1.0-1.2 min); Figure 4-4b and c correspond to the averaged mass spectra obtained during 1.6 to 1.9 min and 2.0 to 2.3 min, respectively, in Figure 4-3.

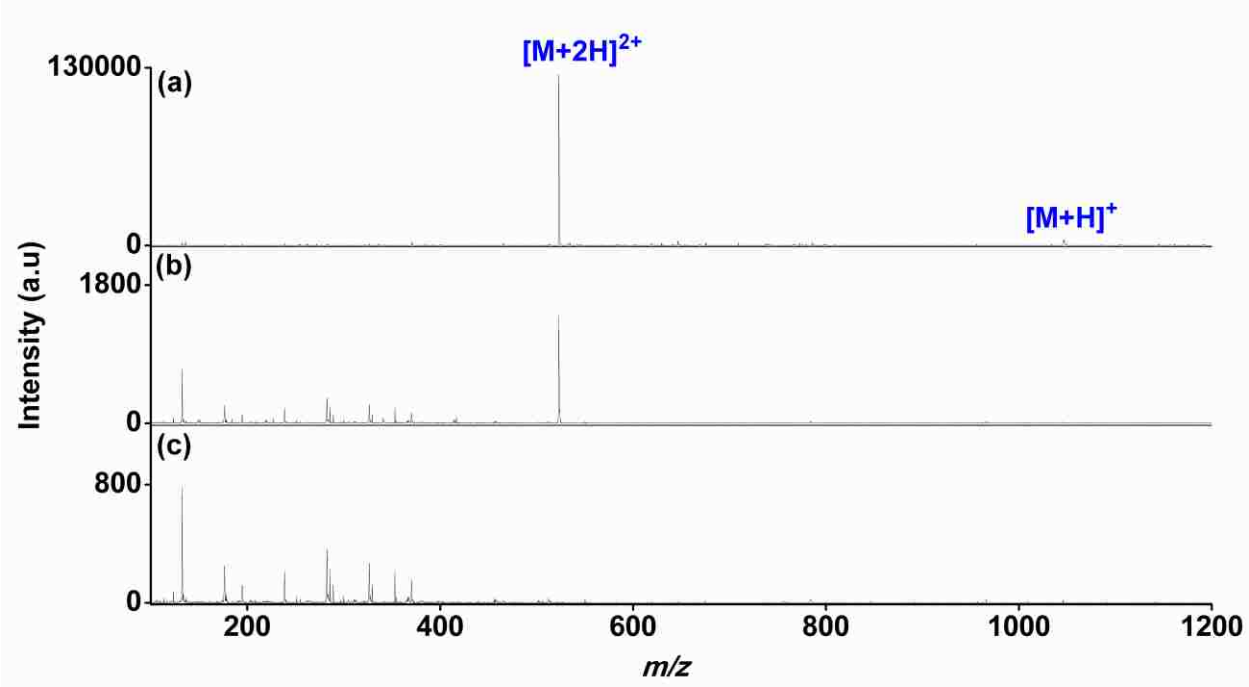

Figure 4-4. Representative mass spectra (corresponding times from Figure 3) obtained during a) 1.0 – 1.2min (laser on), b) 1.6 –1.9min (laser off) and c) 2.0 – 2.3min (laser off).

The sample transfer efficiency can be estimated by comparing the signal obtained by laser ablation capture with that from electrospray of a sample of known concentration. Angiotensin II peptide was deposited on a microscope slide using 20 µL of a 1 mM solution and irradiated with 300 laser shots. The resulting electrospray signal was comparable to that obtained by direct injection of a 1mM solution of the peptide. The 300 µm diameter laser beam irradiated approximately 10^{-3} of the sample spot corresponding to 20 nmol of the peptide. If it is assumed that all of the 20 nmol ablated is captured in a droplet approximately 1 mm in diameter, this will (on dissolution) produce a solution with a concentration of 0.5 mM. This is 50 times as large as the concentration estimated from the observed signal, suggesting a 2% transfer efficiency. In our previous study (see Chapter 3),[17] the estimated transfer efficiency is 1%, comparable to that estimated in this study. This suggests that the droplet capture efficiency is similar in the flowing

and static systems and that there is negligible loss of material between the capture zone and electrospray.

Next, laser ablation sampling of different compounds was tested using sample spots of the peptide angiotensin II and proteins insulin and cytochrome c on the same sample slide. For this experiment, 20 µL volumes of 1 mM standard solutions of angiotensin II, insulin, and

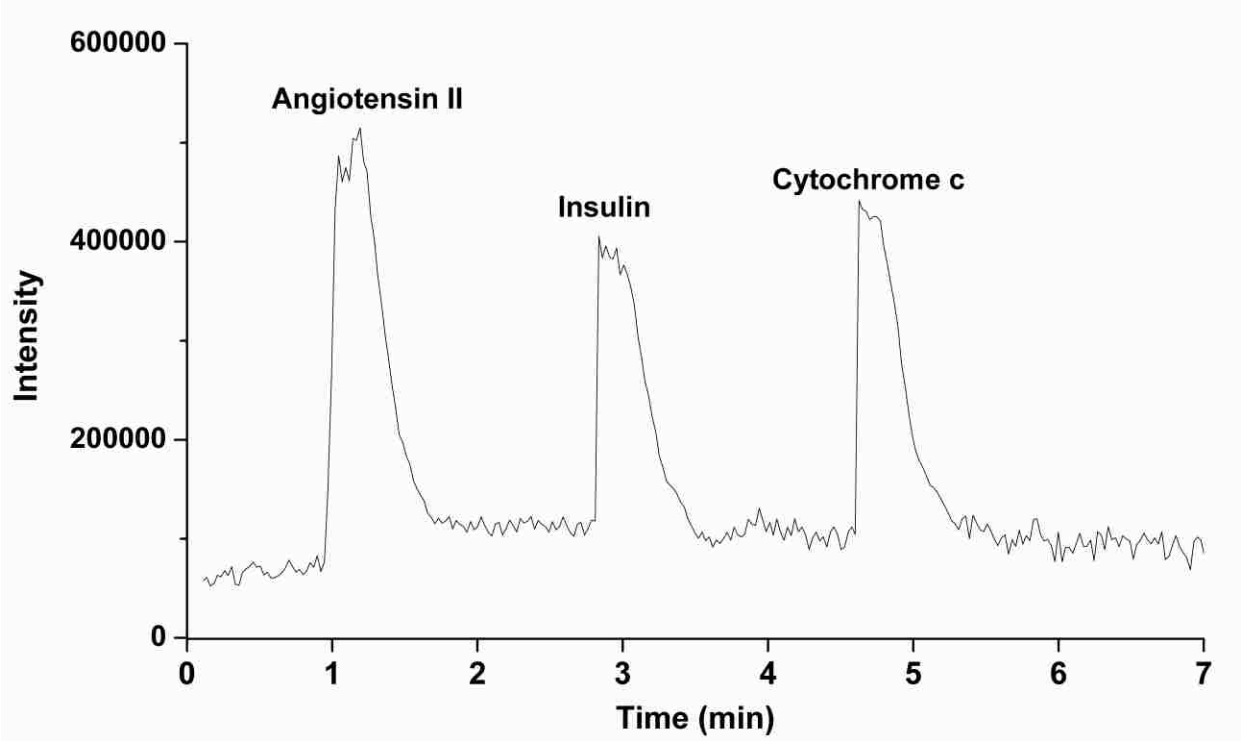

Figure 4-5. TIC from laser ablation of different compounds (angiotensin II, insulin and cytochrome c) on the same sample target.

cytochrome c in 1:1 (v/v) acetonitrile and 0.1% aqueous TFA were deposited in different spots on a microscope slide that was mounted in the translation stage. Laser ablation sampling was accomplished using 150 laser shots at 0.8 mJ of focused laser energy at 2.94 µm wavelength. The TIC plot is shown in Figure 4-5. The memory effect for the protein deposits is similar to that observed for angiotensin II.

Figure 4-6 shows the corresponding mass spectra for the TIC peak of angiotensin II (1.4 min), insulin (3 min) and cytochrome c (5 min). The mass spectrum of angiotensin II in Fig. 6a shows singly charged $[M + H]^{+}$ and doubly charged $[M + 2\ H]^{2+}$ ions. The mass spectrum of bovine insulin in Figure 4-6b shows multiply charged ions from $[M + 7\ H]^{7+}$ to $[M + 4\ H]^{4+}$ and multiply charged cytochrome c ions from $[M + 19\ H]^{19+}$ to $[M + 10\ H]^{10+}$ observed in Figure 4-6c. The mass spectrum from each spot was comparable to those previously obtained using manual injection of droplets containing IR ablated material.[17]

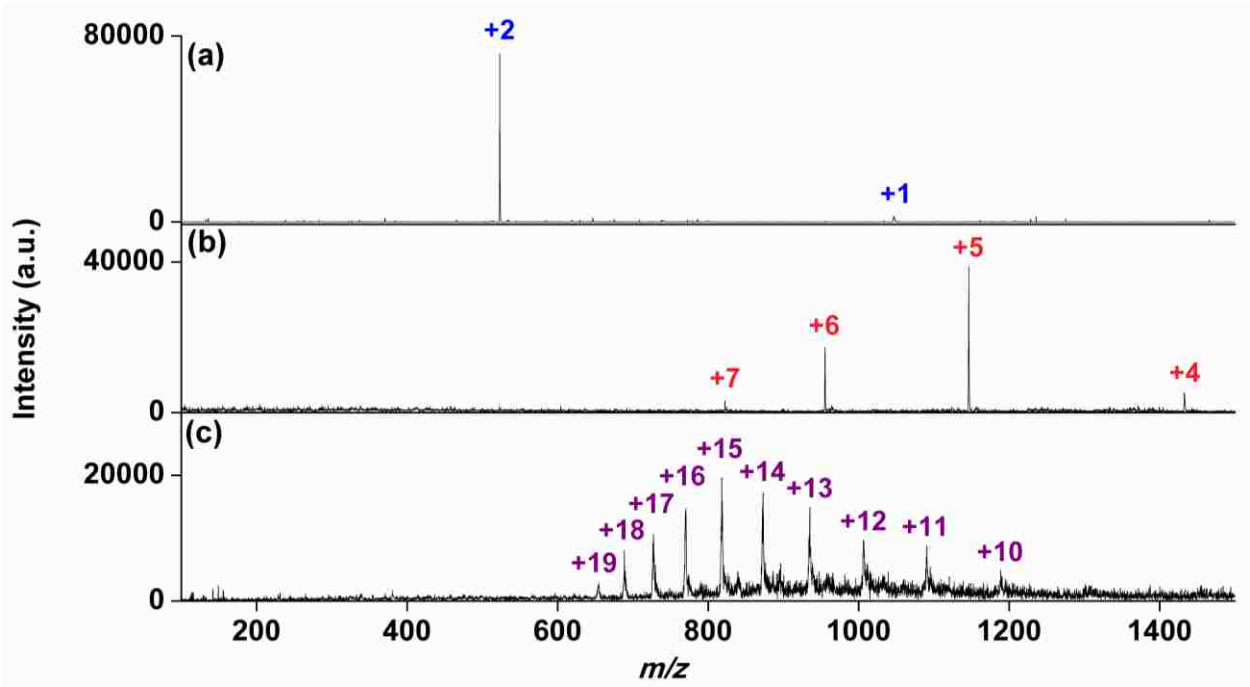

Figure 4-6. Mass spectra for the TIC peaks (in Figure 5) for a) angiotensin II, b) insulin and c) cytochrome c.

Laser ablation capture coupled to LC was demonstrated using a mixture of peptides (angiotensin II, bradykinin and leucine enkephalin) and a mixture of proteins (insulin, myoglobin and lysozyme) deposited in a single spot on microscope slides from 20 mL volumes of mixtures of the 1 mM standard solutions. The mixtures were ablated with 150 laser shots of 0.8 mJ of

focused laser energy at 2.94 µm wavelength. After the sample was ablated for 20 s to allow the captured material to flow into the injection loop, the valve was switched from load to inject to allow the ablated material to enter the LC column. Figure 4-7 shows the TIC chromatograms from the separation of the mixtures: the peptide separation is shown in Figure 4-7a and the

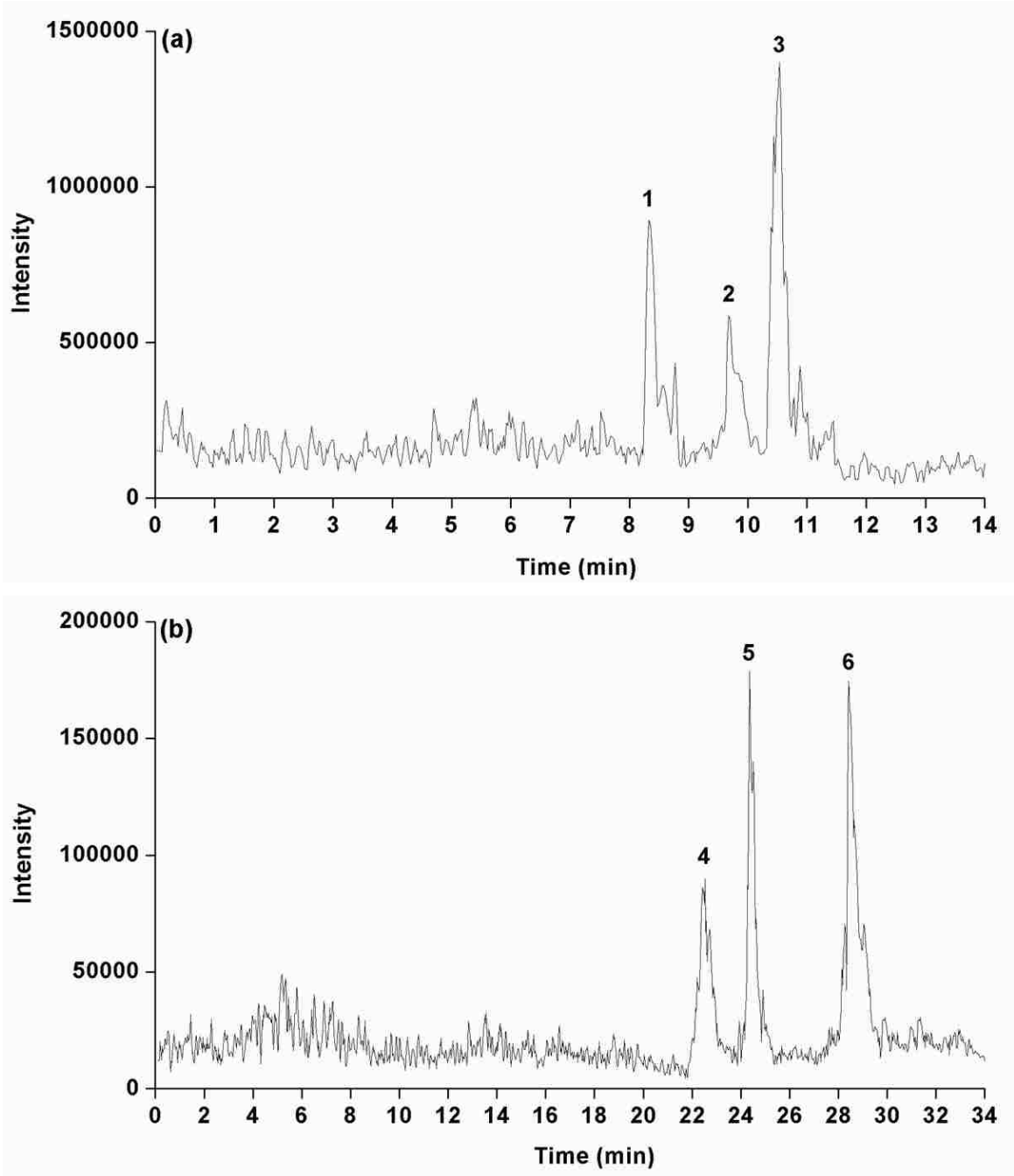

Figure 4-7. TIC from the separation of a mixture of the peptides bradykinin (1), angiotensin II (2) and leucine enkephalin (3) and the proteins insulin (4), myoglobin (5) and lysozyme (6).

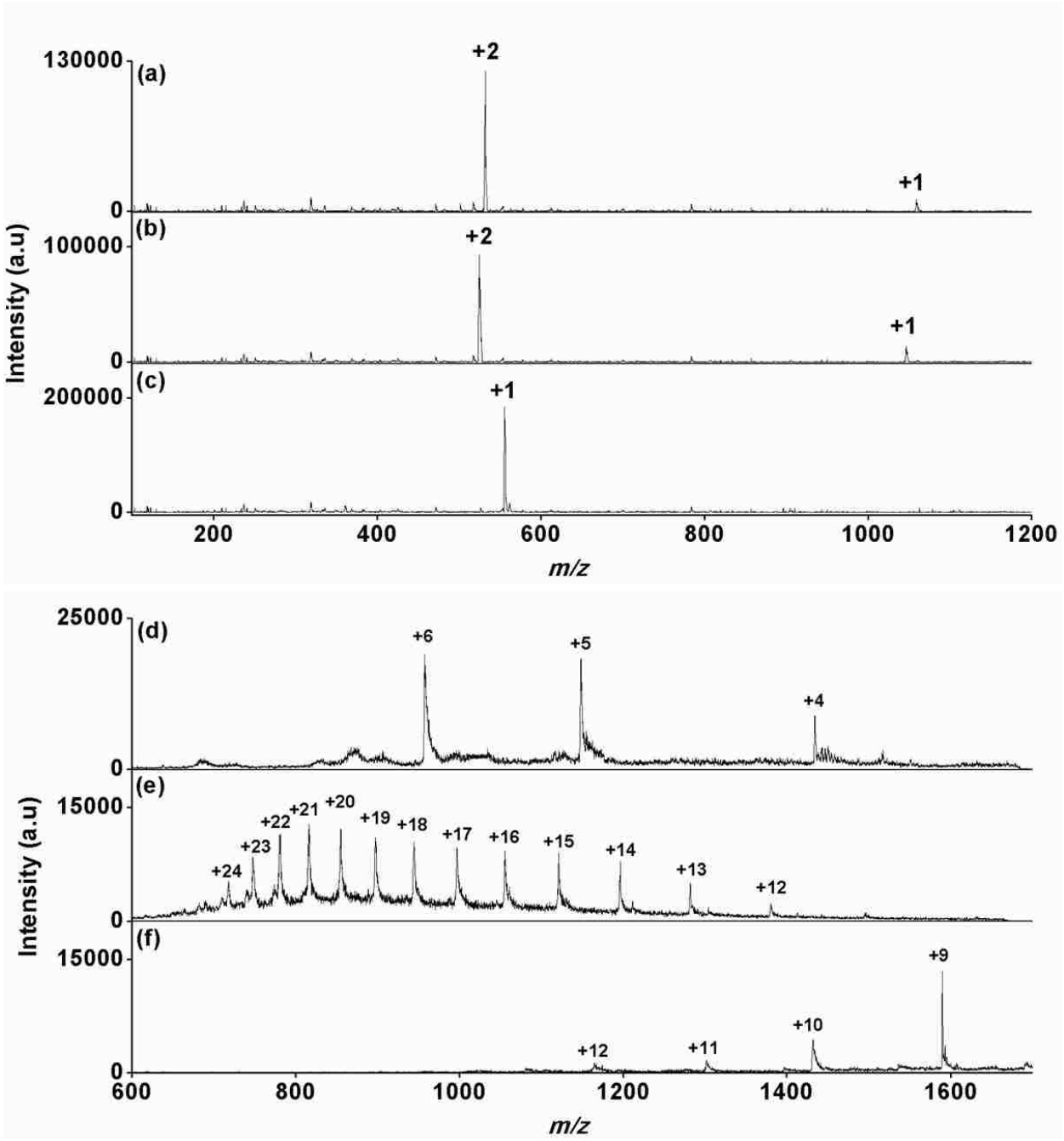

Figure 4-8. Mass spectra corresponding to the TIC peaks in Figure 7 for a) bradykinin, b) angiotensin II, c) leucine enkephalin, d) insulin, e) myoglobin and f) lysozyme.

protein separation is shown in Figure 4-7b. In Figure 4-7a, the three peaks in the TIC chromatogram correspond to bradykinin (Peak 1, 8.5 min), angiotensin II (Peak 2; 9.8 min) and

leucine enkephalin (Peak 3; 10.6 min). In Figure 4-7b, the peaks correspond to insulin (Peak 4; 22.5 min) myoglobin (Peak 5; 24.5 min) and lysozyme (Peak 6; 28.5 min).

Figure 4-8 shows the corresponding mass spectra for each TIC peak of the TIC chromatograms shown in Figure 4-7. The mass spectra of bradykinin (Figure 4-8a) and angiotensin II (Figure 4-8b) show singly charged $[M + H]^+$ and doubly charged $[M + 2\ H]^{2+}$ ions. The mass spectrum of leucine enkephalin (Figure 4-8c) shows singly charged $[M + H]^+$ ion. The mass spectrum of bovine insulin shows multiply charged ions from $[M + 4\ H]^{4+}$ to $[M + 6\ H]^{6+}$ and multiply charged myoglobin ions from $[M +12\ H]^{12+}$ to $[M + 24\ H]^{24+}$ and multiply charged lysozyme ions from $[M + 9\ H]^{9+}$ to $[M + 12\ H]^{12+}$ observed in Fig. 8d-f, respectively.

4.4 Summary

Laser ablation sample transfer into a flowing solvent stream was demonstrated using peptide and protein standard solutions both with direct analysis and with post-ablation separation. A mid-IR laser operating in the 2.94 μm wavelength region was used to ablate material from a sample and transfer it to a flowing solvent stream. A peptide and protein mixture was ablated and separated with LC. A memory effect was observed that led to signal for up to 30 s after the laser was shut off. This could be due to the dead volume of the solvent in the capture zone or delayed solvation of the ablated material at the edge of the capture zone. The efficiency of material transfer from the target to the liquid flow is approximately 2% and comparable to the efficiency observed for transfer to a static liquid drop. Planned improvements and expanded applications for this system include gradient chromatographic separation, laser ablation with microfluidic chip capture, and design of a low dead-volume capture zone with minimal exposed surface area.

The high efficiency of transfer suggests that coupling a laser ablation imaging system with separations and other sample processing steps could prove to be a powerful analytical tool.

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CHAPTER 5. AMBIENT LASER ABLATION SAMPLING FOR CAPILLARY ELECTROPHORESIS MASS SPECTROMETRY*

As is apparent from the results described in the preceding chapters, ambient laser ablation with mass spectrometry detection is a powerful method for direct analysis of biological samples in their native environment. Capillary electrophoresis can separate complex mixtures of biological molecules prior to mass spectrometry analysis and an ambient sampling interface for CE-MS will allow the detection of minor components. For the work described in this chapter, an infrared laser ablated and transferred sample materials under ambient conditions for direct loading onto a CE separation column. Samples were deposited on a transparent target and ablated in transmission geometry using a pulsed mid-infrared laser. The ablated materials were captured in the exposed sampling solvent and then loaded into a capillary by electrokinetic injection for separation and analysis by electrospray ionization (ESI) mass spectrometry. The system was tested using mixtures of peptide and protein standards. It is estimated that tens of fmol of material was transferred from the ablation target and the theoretical plate number was between 1000 and 3000

5.1 Introduction

Capillary electrophoresis has been an important tool for biochemical analysis for over three decades[2] and mass spectrometry has been used as a detector for CE for nearly a quarter century.[3,4] The most often used method for creating ions in CE-MS is electrospray ionization (ESI).[5,6] One of the most challenging aspects of coupling CE to ESI is the electrical connection

* The work reported in this chapter has been published in *Rapid Communications in Mass Spectrometry*.[3] Reprinted by permission of the John Wiley and Sons.

at the spray capillary that must serve as one of the CE electrodes as well as providing the electrospray voltage. There are three general approaches to coupling CE to ESI mass spectrometry: sheathless,[4] sheath flow,[3,7] and liquid junction.[8,9] In the sheathless interface, the electrical contact for the CE and ESI is made with a small diameter wire or conductive coating on the spray tip. The durability and reproducibility of the tips can be a limitation. The sheath flow interface is the most widely used and consists of a tube surrounding the electrospray tip that provides a sheath liquid through which the electrical connection is made. An outer concentric tube is used to provide nebulization gas. The sheath flow interface decouples the CE and ESI voltages but the additional liquid flow dilutes the sample. The liquid junction interface utilizes a small gap near the spray tip to make the electrical connection. Although the liquid junction avoids dilution of the sample, alignment of the capillaries can be difficult.

An alternative to the on-line ESI approach can be achieved by interfacing to matrix-assisted laser desorption ionization either off-line or on-line.[10] Off-line CE-MALDI can be accomplished by blotting onto a membrane,[11] fraction collection with a sheath flow,[12,13] sheathless,[14] or liquid junction configuration.[15] Co-deposition with matrix can be accomplished by spotting CE effluent and matrix onto the target,[16] or by depositing the effluent track on a moving target for readout in the mass spectrometer.[13,17,18] On-line coupling of CE to MALDI has been accomplished by depositing the capillary effluent onto a rotating wheel[19] or rotating ball[20] that serves and the MALDI target in vacuum. The rotating ball interface has been used to couple an electrokinetically driven microfluidic chip to MALDI.[19] Capillary electrophoresis chips have been coupled off-line to MALDI.[21,22]

The main difficulty to on-line interfacing CE with MALDI is that the sample must be delivered from ambient conditions to vacuum. Ambient mass spectrometry can overcome this limitation.

Ambient ionization has been coupled with various separation methods, such as thin layer chromatography (TLC), liquid chromatography (LC), and CE, which allows the direct analysis of separation media or effluent without further processing (see Chapter 1).[23-33]

Laser ablation can also be used for ambient sample loading prior to separation and ionization for mass spectrometry. Laser ablation sampling for LC has been demonstrated using visible,[34] and infrared lasers described in Chapter 4. Here, the sample is ablated by the laser and captured in a liquid flow. The plug of sample is injected into the column where the components are separated and ionization occurs by electrospray after the separation.

For the work described in this chapter, ambient laser ablation sample transfer was coupled to capillary electrophoresis separation of the transferred analyte. Electrospray ionization mass spectrometry was used to detect the separated components. Coupling ambient IR laser ablation on-line sampling to capillary electrophoresis electrospray ionization mass spectrometry (CE-ESI/MS) required the development of a novel collection and electrokinetic transfer system. Materials deposited on a sample slide were irradiated by an IR laser to transfer materials to the exposed sampling solvent. The ablated material was captured in the solvent and loaded into a separation capillary by electrokinetic injection and the separated material was analyzed using ESI/MS.

5.2 Experimental

The configuration depicted in Figure 5-1 shows the schematic for off- and on-line laser ablation sample transfer collection and electrokinetic sample manipulation for CE-ESI/MS that was used in this work. The system consisted of a laser ablation sampling collection zone and a CE separation zone. The CE was a home-made system that utilized two 30 kV voltage regulated power supplies. The on-line laser ablation sampling zone was based on the LC collection described in Chapter 4 that was modified for electrokinetic rather than pressure driven flows.

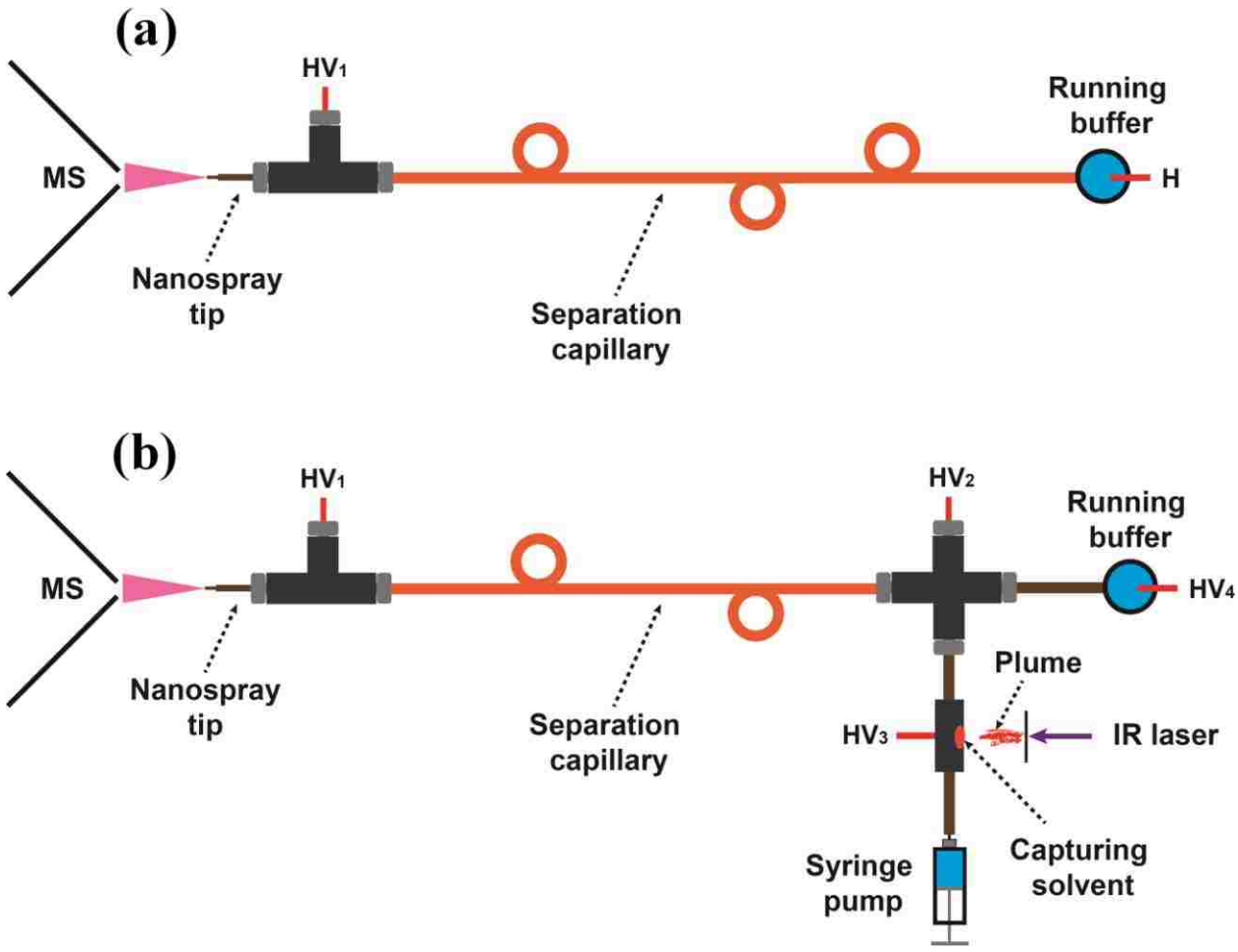

Figure 5-1. Schematic of the laser ablation sample transfer collection systems for a) off-line CE/ESI and b) on-line CE/ESI.

Briefly, samples were deposited in a 20 µL volume to form a spot 8 mm in diameter on an indium tin oxide coated microscope slide mounted on the 3-axis translation stage (M-433, Newport). The slide was held 1 mm from the open solvent channel with the sample facing the channel. The solvent channel was constructed using a poly(vinyl alcohol) (PVA) coated capillary (360 µm O.D. × 50 µm I.D. × 5 cm), an uncoated fused silica capillary (360 µm O.D. × 50 µm I.D. × 10 cm) and a PEEK tubing sleeve (1.57 mm O.D. × 0.39 mm I.D.). The PEEK tubing was notched half-way through the diameter to expose a 1 mm section longitudinally for the capture zone. A 0.2 mm dia. platinum wire (HV_3) was inserted through a hole in the PEEK tube opposite the capture zone and the hole was then sealed with epoxy. The injection of the ablated material into the separation capillary was based on tee injection developed for microchip CE-MS.[35-37]

The PVA-coated and uncoated capillaries were inserted into the PEEK tube with a 1 mm spacing between them at the capture zone. The end of the uncoated capillary was connected to a syringe pump (11 Plus, Harvard Instruments, South Natick, MA, USA) to supply solvent (1% acetic acid for peptide and 0.1% acetic acid for protein[38,39]) to the open channel. The end of the PVA-coated capillary was connected to a 4-way cross union (Upchurch, Oak Harbor, WA, USA; 360 µm O.D. tubing and 152 µm thru hole). The remaining channels of the cross union were connected to the separation capillary and running buffer reservoir. A platinum wire was used to electrokinetically control the sample flowing during sample injections and separations (HV_2). The end of a PVA-coated capillary (360 µm I.D. × 50 µm I.D. × 10 cm) was placed in a running buffer reservoir (HV_4). The PVA-coated separation capillary (360 µm I.D. × 50 µm I.D.) was connected between the 4-way cross union and a 3-way cross union with a 152 µm through hole (Upchurch). The 3-way cross was held an uncoated electrospray emitter that was 360 µm O.D. × 50 µm I.D. with a 15 µm tip I.D. (SilicaTip, New Objective, Woburn, MA, USA). To interface

the CE to ESI, a liquid junction interface[40] was used with a 0.2 mm platinum wire that was inserted into the side channel of the 3-way cross union to supply the potential for electrospray (HV_1). The length of the separation capillary was 55 cm for peptides and 80 cm for proteins.

The electrospray voltage was set to 2.0 kV. The tee unions and nanospray tip were mounted on the 3-axis stage and positioned 2 mm from the mass spectrometer sampling cone. The samples were ablated in transmission geometry with the IR OPO used to transfer the ablated material to the exposed sampling solvent. The wavelength of the laser was set at 2.94 μm and the focused laser spot was 300 μm in diameter.[41,42] Mass spectra and total ion electropherograms (TIE) were acquired using the ion trap mass spectrometer. Mass spectra were acquired in positive-ion mode and ions were accumulated for 70 ms in the trap. No nebulizer gas was used.

Coating of silica capillaries with PVA was performed using the procedure described by Belder et al.[43] This procedure minimizes analyte interactions with the negatively charged silanol surface groups under acidic conditions that lead to poor reproducibility of CE migration times, resolution, and peak shapes, especially for proteins.[43-48] The fused silica capillaries (Polymicro Technologies, Phoenix, AZ, USA) were filled with an acidified aqueous glutaraldehyde solution (200 μL of a 50% aqueous solution mixed with 300 μL of 1:10 diluted conc. HCl). After that, an acidified PVA solution (450 μL of a 5% aqueous PVA solution, mixed with 50 μL of 1:1 H_2O diluted HCl) was passed through the column with a syringe pump for 10 s. The capillary was then emptied and dried under nitrogen flow for 10 min. The capillary was placed in a GC oven and, while flushed with nitrogen, the oven was heated from 40 to 160 ^oC at 6 ^oC/min.

The peptide standards bradykinin, neurotensin, angiotensin II, and leucine enkephalin, and protein standards bovine insulin, myoglobin, lysozyme, and reagent acetic acid were used without further purification. House ultrapure water (18 MΩ cm) was used. Analyte solutions

were prepared by dissolving the standards in 1% acetic acid (pH = 2.8) solution for peptides and 0.1% acetic acid (pH = 3.3) for proteins to a final concentration of 1 mM. For the ablation transfer quantification studies, the peptide mixture solution was serially diluted in the 1% acetic acid solution from 100 µM to 1 µM.

5.3 Results

Initial testing of the system was accomplished using off-line CE-MS with laser-ablated material captured in a solvent droplet and manually injected into the separation capillary as shown in Figure 5-1a. Here, 20 µL of a solution containing 1 mM of bradykinin, neurotensin, angiotensin II and leucine enkephalin was deposited on a sample slide. The sample was ablated with 300 laser shots that transferred the material to a 1% acetic acid solvent droplet suspended 1 mm from the slide. The solvent droplet was then transferred to a microcentrifuge tube. A 55 cm separation capillary was flushed with a 1% acetic acid solution then immersed in the microcentrifuge tube for electrokinetic sample injection at 5 kV for 5s. The sample volume was approximately 15 nL and, assuming the 2% transfer efficiency that we estimated in a previous study.[49] This corresponds to about 20 pmol of each peptide injected.

For separation, the capillary inlet was moved to a running electrolyte reservoir filled with the 1% acetic acid solution and 14 kV was applied to the reservoir with a platinum wire. The voltage applied to the electrospray tip was set at 2 kV during sample injection and separation. The resulting TIE obtained in full-mass scan acquisition is presented in Figure 5-2a. The electropherogram shows four resolved peaks; bradykinin (8.9 min), neurotensin (10.7 min), angiotensin II (11.9 min) and leucine enkephalin (14.9 min). The theoretical plates for the separation are bradykinin, 2000; neurotensin 2800; angiotensin II, 2700 and leucine encephalin,

4400. The resolution is likely limited by the dead volume in the tee holding the nanospray tip and by the alignment of the tip in the liquid junction.

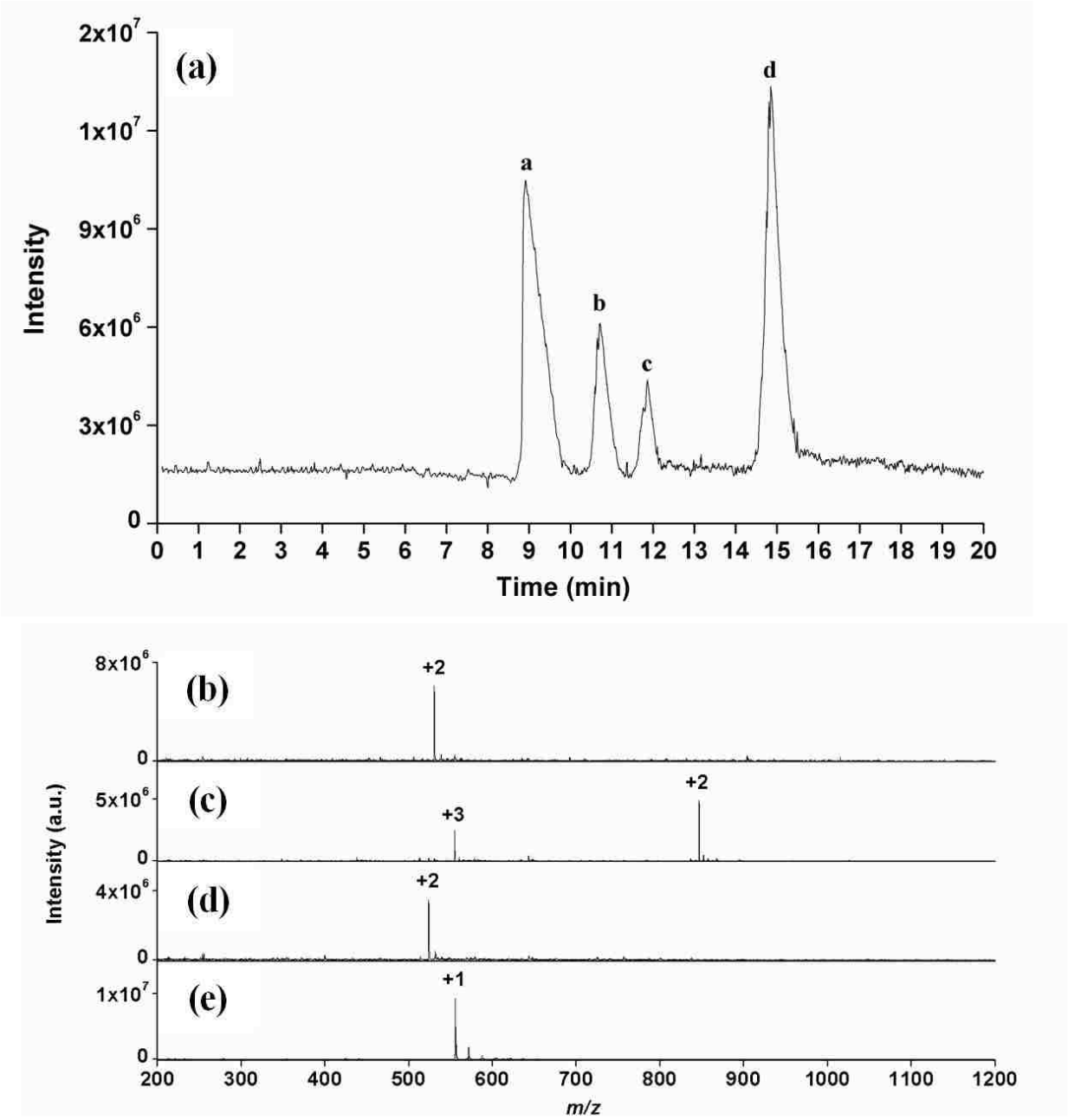

Figure 5-2. (a) Total ion electropherograms (TIEs) from manual injection of IR laser-ablated material and corresponding mass spectra for (b) bradykinin (8.9 min), (c) neurotensin (10.7) min, (d) angiotensin II (11.9 min), and (e) leucine enkephalin (14.9 min).

The peptide mass spectra for each peak on the TIE are shown in Figure 5-2. The mass spectra of bradykinin (Figure 5-2b) and angiotensin II (Figure 5-2d) show doubly charged $[M + 2H]^{2+}$ ions. The mass spectrum of neurotensin (Figure 5-2c) shows triply charged $[M + 3H]^{3+}$ and doubly charged $[M + 2H]^{2+}$ ions. The mass spectrum of leucine enkephalin (Figure 5-2d) shows singly charged $[M + H]^{+}$ ion.

For on-line sampling with CE-MS, the tee interface was used to inject the ablated material that was captured in the sampling solvent into the separation capillary and the liquid junction interface was used to couple the CE and ESI. To demonstrate peptide transfer, a 20 μL volume of the 1 mM peptide mixture was deposited on a microscope slide and irradiated in transmission mode using 300 IR laser shots. The captured material was then injected into the separation capillary by applying 8 kV to HV_3 (see Figure 5-1a) for 15 seconds. At this point, 5

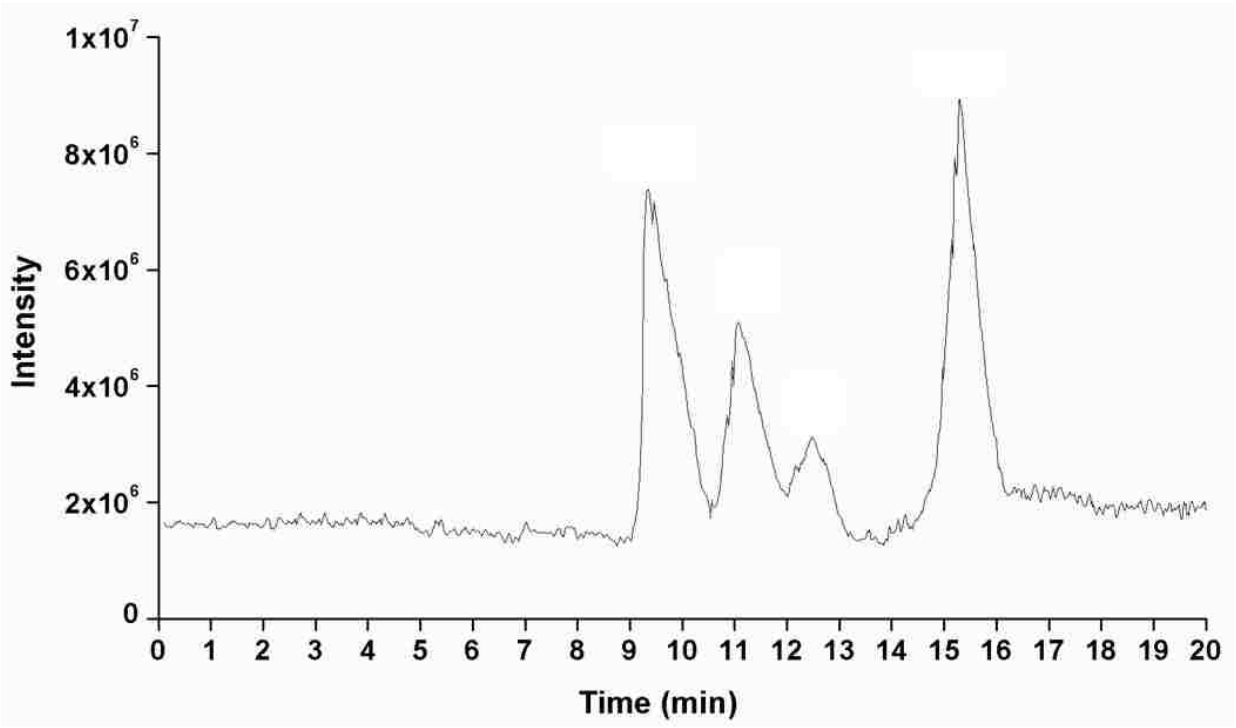

Figure 5-3. Total ion electropherograms (TIEs) from on-line injection of IR-laserablated material and corresponding mass spectra for (a) bradykinin, (b) neurotensin, (c) angiotensin II, and (d) leucine enkephalin.

kV was applied to the running electrolyte reservoir at HV_4 and 5 kV to the tee at HV_2 with 2 kV at the electrospray needle (HV_1). After sample injection, HV_3 and HV_2 were switched to 14 kV, while HV_4 was increased to 17 kV.

Figure 5-3 shows the resulting TIE. The four peptides are clearly separated with bradykinin (9.4 min), neurotensin (11.1 min), angiotensin II (12.5 min) and leucine enkephalin (15.3 min). The integrated peak areas from on-line sampling were nearly the same as those from manual injection (Figure 5-3) suggesting that the on-line and off-line transfer efficiency is comparable and that on-line transfer led to approximately 20 pmol of each peptide injected into the separation capillary. The theoretical plates for the separation are bradykinin, 1100; neurotensin 1600; angiotensin II, 1400 and leucine encephalin, 2900. The resolution of the CE separation obtained with on-line transfer is two-thirds that of the off-line transfer, most likely due to dead volume in the tee junction between the sampling zone and the separation capillary.[50]

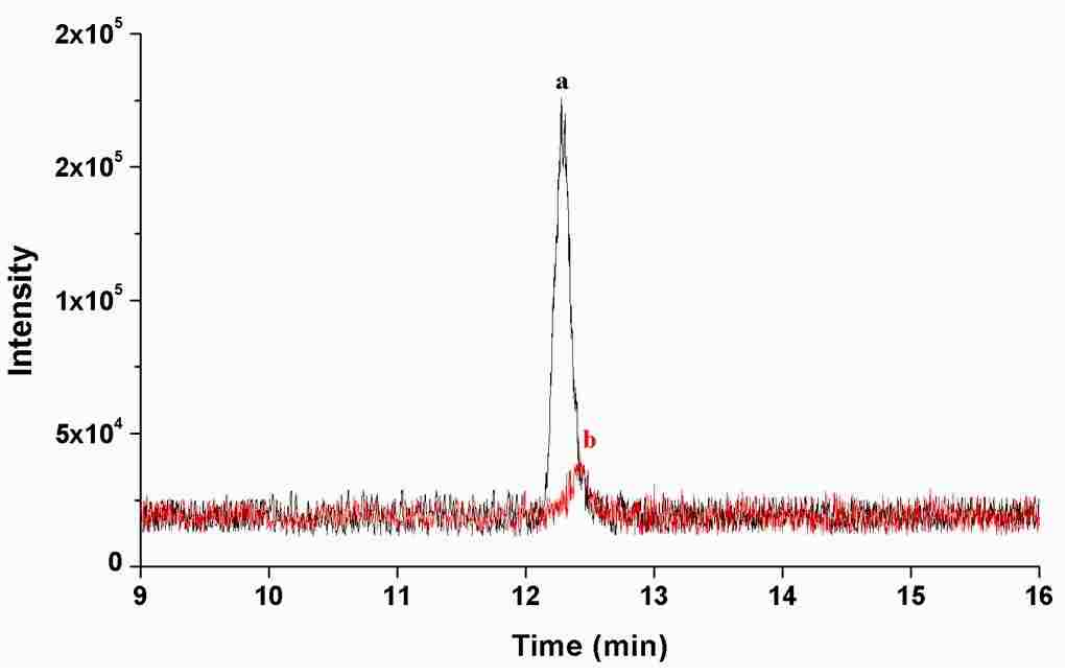

Figure 5-4. Representative TIEs from on-line injection of a laserablated material for doubly charged angiotensin II: (a) 200 and (b) 20 fmol transferred and injected into the CE system.

To test reproducibility in migration times, a peptide mixture was on-line injected using IR laser transfer. All experimental conditions for laser ablation, sample injection, and separation were the same as those for the on-line laser ablation sampling shown in Figure 5-4. The relative standard deviation (RSD) values for the migration times were less than 3% for four on-line transfer injections.

The ability to detect transferred material for the system in terms of the quantity of solid sample ablated was investigated in selected ion monitoring (SIM) mode using a peptide standard solution containing bradykinin, neurotensin, angiotensin II, and leucine encephalin. The limit of detection was taken as three times signal-to-nose (S/N). For this experiment, a peptide mixture was prepared by serial dilution in a 1% acetic acid solution with final concentrations ranging from 1 to 100 µM. A 20 µL volume of the diluted solution was deposited on the sample slide and IR laser ablated. The transferred material was injected and separated for ESI/MS in SIM mode. The experimental conditions used for laser ablation, injection, and separation were the same as above. Examples of SIM for the ablation of material deposited from a) 10 µm and b) 1 µm angiotensin II solutions, respectively, are shown in Figure 5-5. The quantity transferred at three times signal-to-nose (S/N) is estimated to be 2 pmol of angiotensin II assuming complete ablation of the material in the 300 µm laser spot. A 2% sampling efficiency was estimated previously for capture of IR ablated material in a static droplet and, assuming a similar capture efficiency for the current system, it can be estimated that 40 fmol of angiotensin II was injected into the CE capillary. The transferred for the other peptides in SIM mode was estimated to be 15 fmol for bradykinin, 20 fmol for neurotensin, and 10 fmol for leucine enkephalin.

The system was further evaluated using a mixture of proteins using an 80 cm PVA-coated capillary. A 20 µL volume of the protein mixture, containing 1 mM each of insulin, lysozyme,

and myoglobin, was ablated by 300 laser shots at 2.94 μm wavelength and transferred to the exposed sampling solvent. The captured compounds were then injected into the separation capillary by applying 8 kV to HV_3, 5 kV to HV_4, and HV_2 for 15 seconds with 2 kV on HV_1 at

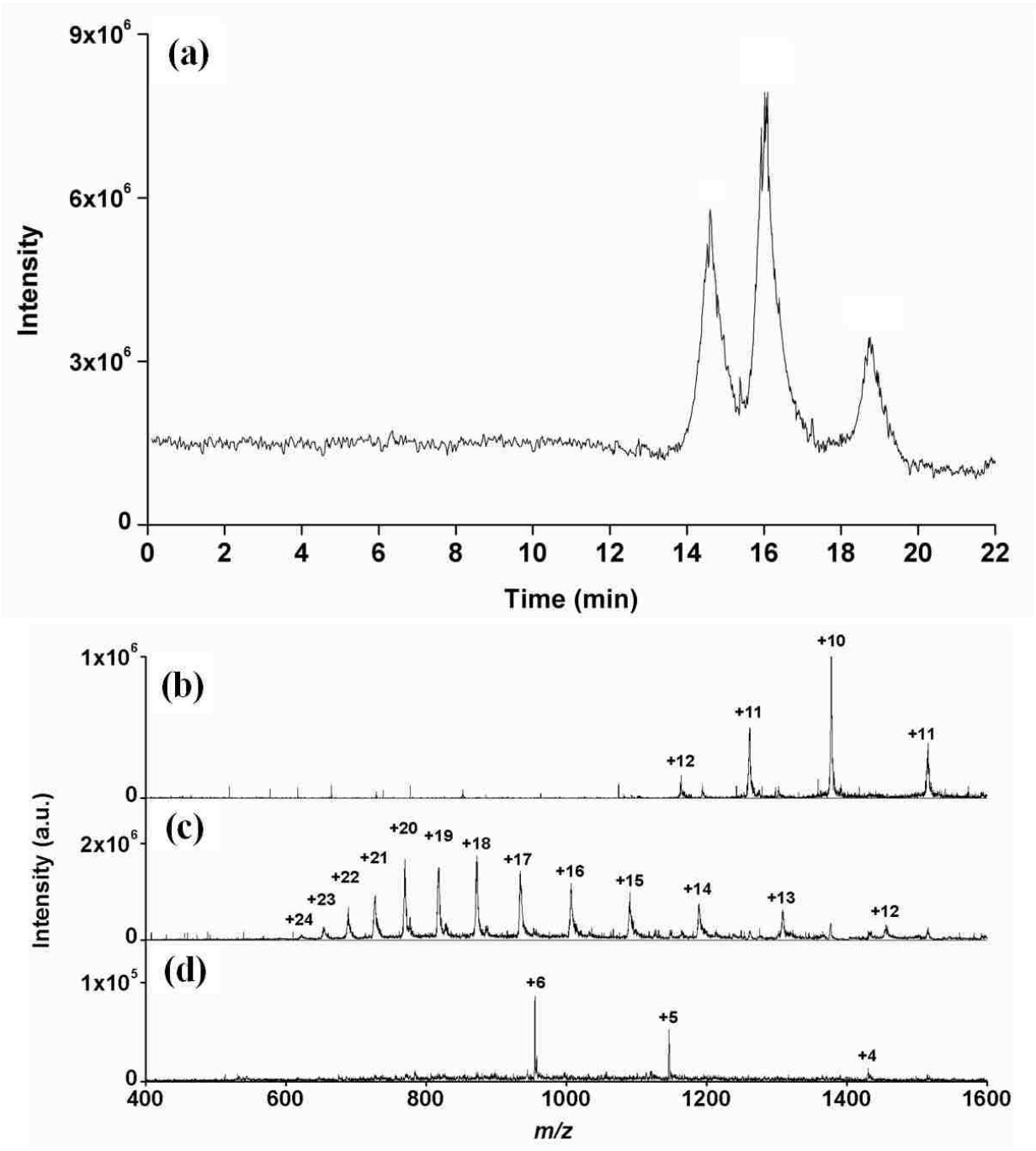

Figure 5-5. (a) Total ion electropherograms (TIEs) from on-line injection of IRablated material and corresponding mass spectra for (b) lysozyme (14.6 min), (c) myoglobin (16.0 min), and (d) insulin (18.7 min).

the electrospray tip. After injection, the applied voltages to HV_3 and HV_2 were switched to 22 kV, while 25 kV to HV_4 and the electrospray tip HV_1 remained at 2 kV. The running electrolyte used for separating the protein mixture was 0.1% acetic acid. The resulting TIE shown in Figure 5-5a has three peaks; lysozyme (14.6 min), myoglobin (16.0 min) and insulin (18.7 min). The corresponding mass spectra for each peak of the electropherogram are shown in Figure 5-5b-d. The mass spectra show multiply charged lysozyme from $[M + 9\ H]^{9+}$ to $[M + 12\ H]^{12+}$ and multiply charged myoglobin from $[M + 12\ H]^{12+}$ to $[M + 24\ H]^{24+}$ and multiply charged insulin from $[M + 4\ H]^{4+}$ to $[M + 6\ H]^{6+}$ observed in Figure 5-5b-d, respectively.

We anticipate that the utility of laser ablation coupled with CE-MS will be realized in imaging mass spectrometry. Small molecule imaging with laser ablation capture and liquid chromatography separation has been demonstrated recently.[51] Extending this approach to large biomolecules and chip-based electrophoretic separations will enhance the ability to perform imaging of minor components in biological samples. Further, the approach is general: the laser ablated material can be captured on a microfluidic chip for manipulation and analysis in a number of ways prior to mass spectrometry or with analysis methods in addition to or instead of MS.

5.4 Summary

An on-line ambient CE-MS sampling system based on IR laser ablation sample transfer was demonstrated using peptide and protein mixtures. A mid-IR OPO laser system operating at 2.94 μm ablated solid samples that were transferred to solvent that was injected into a CE separation capillary for ESI-MS. With this system, the ablated peptide and protein mixtures were captured, injected, and each peptide and protein was resolved. The number of theoretical plates

for the peptides ranged from 1100 to 2900 and the resolution was between 1 and 2. The on-line injection resolution was about two-thirds the off-line resolution, possibly due to dead volume in the connection between the injection and separation zones. LODs were 15 fmol for bradykinin, 20 for neurotensin, 40 for angiotensin II and 10 for leucine enkephalin. Reproducibilities (n=4) for migration times were 1.4% for bradykinin, 2.3% for neurotensin, 2.9% for angiotensin II and 1.1% for leucine encephalin.

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CHAPTER 6. LASER ABLATION SAMPLE TRANSFER FOR MALDI IMAGING*

For the work described in this chapter, an infrared OPO was used to ablate material from tissue sections under ambient conditions for direct collection on a matrix assisted laser desorption ionization (MALDI) target. A 10 μm thick tissue sample was placed on a microscope slide and was mounted tissue-side down between 70 and 450 μm from a second microscope slide. The two slides were mounted on a translation stage, and the tissue was scanned in two dimensions under a focused mid-infrared (IR) laser beam to transfer material to the target slide via ablation. After the material was transferred to the target slide, it was analyzed using MALDI imaging using a tandem time-of-flight mass spectrometer. Images were obtained from peptide standards for initial optimization of the system and from mouse brain tissue sections using deposition either onto a matrix pre-coated target or with matrix addition after sample transfer and compared with those from standard MALDI mass spectrometry imaging. The spatial resolution of the transferred material is approximately 400 μm. Laser ablation sample transfer provides several new capabilities not possible with conventional MALDI imaging including (1) ambient sampling for MALDI imaging, (2) area to spot concentration of ablated material, (3) collection of material for multiple imaging analyses, and (4) direct collection onto nanostructure assisted laser desorption ionization (NALDI) targets without blotting or ultrathin sections.

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6.1 Introduction

Imaging mass spectrometry using matrix assisted laser desorption ionization (MALDI) has seen rapid growth since its introduction 16 years ago.[2] MALDI imaging has been primarily applied to the analysis of biomolecules in tissue sections and has been used to investigate small molecules such as pharmaceuticals and their metabolites as well as large biomolecules such as proteins.[3-6] In a MALDI imaging experiment, the tissue of interest is cut into an approximately 10 μm thick section that is deposited on a conductive microscope slide. The matrix is added by dropping, spraying, or other means, and the slide is mounted in a specially designed holder for insertion into the vacuum of the mass spectrometer. The image is obtained by recording mass spectra at an array of evenly spaced positions on the slide and displaying the signal intensity for an ion of a particular mass as a function of the position. The spatial resolution is limited by the spot size of the focused laser to approximately 25 μm. Higher spatial resolution can be achieved using specially designed microprobe[7] or near field optics,[8] but such approaches have not been adapted to commercial instruments. The throughput requirements of MALDI imaging have prompted a move from the 337 nm nitrogen laser to solid state lasers such as the frequency tripled Nd:YAG lasers operating at 355 nm wavelength.[9] Current commercial instruments operate with 1 kHz repetition rate lasers.

One of the most critical components of MALDI imaging is the addition of the matrix to the tissue sample.[10] The matrix must be applied in such a way as to promote the cocrystallization with the analyte molecules in the tissue while at the same time maintaining the lateral localization of the analyte to preserve the spatial resolution in the resulting image. The deposition of individual matrix droplets, either manually or with an automated spotter, is adequate for coarse resolution imaging, known as "profiling mode".[11] A pneumatic spray such as

that from a thin layer chromatography (TLC) sprayer can produce excellent matrix and analyte interaction while at the same time good spatial resolution ("imaging mode").[12] Dry powder matrix addition is particularly effective for matrix and analtye materials that are not soluble in standard solvents,[13] and sublimation addition of matrix is another solvent-free method for MALDI imaging.[14] Imaging can also be performed on nanostructured targets. A nanostructured porous silicon DIOS target was used for imaging of 50 nm thick tissue sections.[15] The extremely thin sections were necessary to avoid covering the nanostructures with tissue. An alternate approach is to blot the tissue onto the surface and then remove it for analysis. Imaging of lipids blotted onto a commercial nanostructure assisted laser desorption ionization (NALDI) target has been reported.[16]

Although imaging using a conventional MALDI mass spectrometer is a powerful tool, there are advantages to imaging under ambient conditions, which can be accomplished with a number of ambient ionization methods.[17-21] MALDI itself can be performed under ambient conditions, which obviates the need to deliver the sample to vacuum but still requires the addition of matrix.[22] With desorption electrospray ionization, a spray of charged droplets is directed at the sample to extract analyte that is subsequently removed and ionized. Imaging is accomplished by scanning the spray across the tissue.[23] With a surface sampling probe, the material is extracted directly from the surface for electrospray ionization.[24] Lasers can be used to ablate material that interacts with the electrospray plume for ionization, for example, in electrospray laser desorption ionization (ELDI)[25] and matrix-assisted laser desorption electrospray ionization (MALDESI).[26] Infrared lasers are highly efficient at material removal by ablation and have been used to some advantage in laser ablation based ambient ionization

methods.[27-30] Laser ablation can also been used to assist surface sampling with either MALDI[31] or electrospray ionization.[32]

In this chapter, we report on the adaptation of IR laser ablation sample transfer to MALDI tissue imaging. The IR laser was used in transmission mode to ablate material from a microscope slide for capture on a second slide a fraction of a millimeter away. The approach was optimized using deposits of peptide mass standards and demonstrated with mouse brain tissue sections. The ability to sample under ambient conditions and image under vacuum was demonstrated. We also report on direct transfer to a NALDI target for imaging, multiple transfers from a single tissue section, and sample concentration by ablation from a large area onto a single target spot.

6.2 Experimental

IR laser ablation/capture approach described in the previous chapters was adapted to laser ablation sample transfer from a tissue sample to a MALDI target as shown in Figure 6-1. This system consisted of two indium tin oxide (ITO) coated microscope slides that were mounted on the two-dimensional translation stage (M-433, Newport) operated using computer-driven actuators (LTAHS, Newport) and a motion controller (ESP300, Newport). The sample was deposited or tissue section mounted on one microscope slide (sample slide) that was placed against a second slide (target slide) or a NALDI target. The sample side faced downward toward the target, and the gap between the slide and target was adjusted using different thicknesses of adhesive tape; the spacing was determined using a measuring microscope. Thicknesses of 450, 120, and 70 μm were achieved.

The MALDI target was coated with a thin layer of nitrocellulose by spraying a solution of nitrocellulose in a methanol and acetone 4:1 (v/v) solvent using a TLC sprayer (Z529737, Sigma-Aldrich). For matrix precoated targets, either 2,5-dihydroxybenzoic acid (DHB) or sinapinic acid (SA), used for mouse brain tissue imaging, or α-cyano-4-hydroxycinnamic acid (CCA), used for angiotensin II, were sprayed onto the MALDI target after the nitrocellulose. Ten coats each of the nitrocellulose and matrix solutions were applied using a 20 s spray coating followed by a 2 min interval for drying.

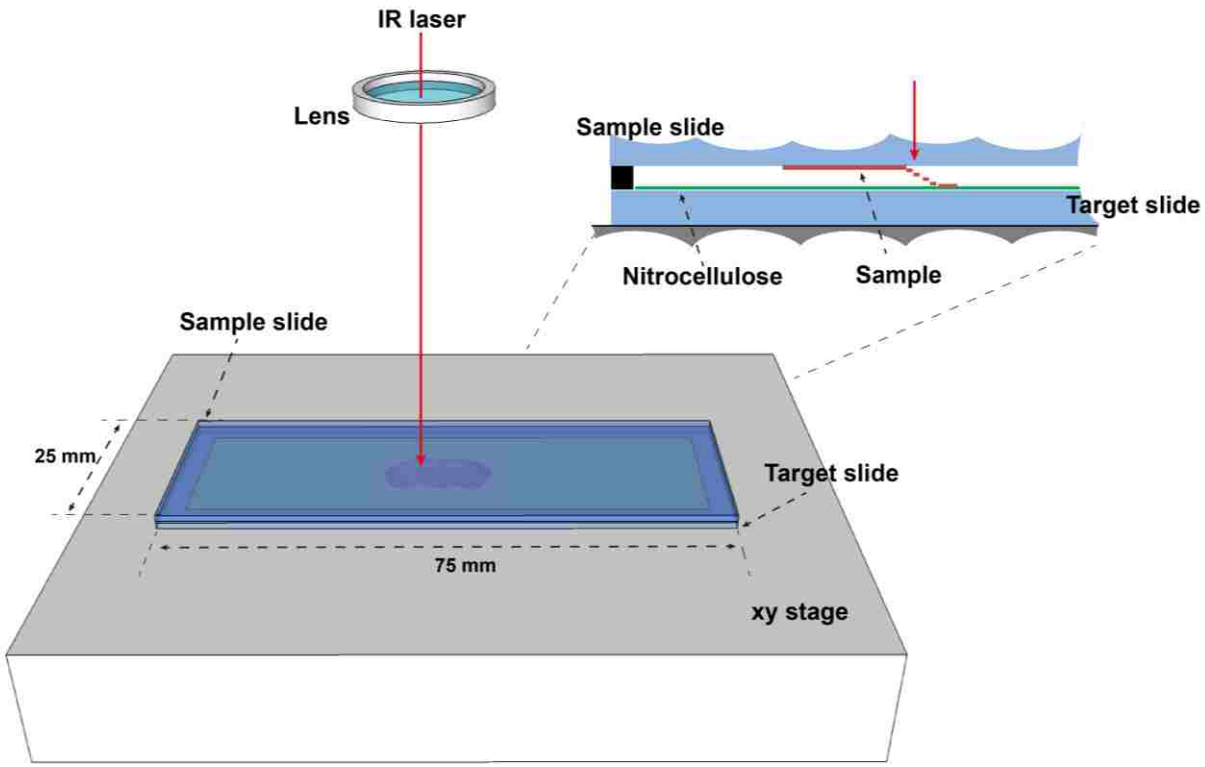

Figure 6-1. Schematic of the laser ablation sample transfer system for MALDI imaging.

Samples were ablated in transmission geometry using the wavelength tunable pulsed infrared optical parametric oscillator. The laser was directed at the sample target at a 90° angle and was focused onto the sample with a 50 mm focal length lens. The pulse width was 5 ns, the repetition rate was 20 Hz, and the wavelength was set at 2.94 μm to overlap with the OH stretch

absorption of the analyte.[33,34] The spot size of the laser beam at the sample was approximately 300 μm × 200 μm as determined using laser burn paper. The maximum laser energy was 2.0 mJ, corresponding to a fluence of 30 kJ/m^2.

The sample slide and MALDI target were scanned under the IR laser beam to transfer material from the sample slide to the MALDI target. The linear velocity of the stage was 30 μm/s, and a serpentine pattern with 20 μm raster line spacing was traced. After the transfer of the laser ablated material to the target, mass spectra were obtained using MALDI mass spectrometry.

Mass spectra were acquired using the Bruker Ultraflextreme MALDI-TOF mass spectrometer in reflectron mode. Raster scans on sample surfaces were performed in imaging mode with 300 shots per sample spot with a step size of 100 μm and a laser spot size of 100 μm. Ion images were reconstituted using FlexImaging 2.0 software.

The peptide standard angiotensin II, reagents trifluoroacetic acid (TFA) and acetone, and matrix compounds CCA, DHB, and SA were used without further purification. Nitrocellulose was purchased from Schleicher & Schuell (Dassel, Germany). HPLC grade methanol, acetonitrile (ACN), and glycerol were purchased from Fisher Scientific (Pittsburgh, Pennsylvania). House ultrapure water (18 MΩ cm, Barnstead E-pure System; Dubuque, Iowa) was used. A 1 mM solution of angiotensin II was prepared by dissolving in 1:1 (v/v) methanol and 0.1% aqueous TFA. A saturated matrix solution for the angiotensin II was prepared by dissolving 50 mg/mL of CCA in a 1:1 (v/v) mixture of methanol and 0.1% aqueous TFA. The matrix solution for the mouse brain tissue was prepared by dissolving 35 mg/mL of DHB in a 1:1 (v/v) mixture of methanol and 0.1% aqueous TFA. The SA matrix was dissolved in ACN and 0.2% TFA at 3:2 (v/v). The nitrocellulose solution was prepared by dissolving 100 mg in a 20 mL methanol and acetone solution at 4:1 (v/v). A glycerol solution was prepared by dissolving

glycerol in methanol and 0.1% aqueous TFA mixture solution in 1:1 (v/v) ratio.

6.3 Results

For initial tests, two MALDI targets were prepared to collect the laser-ablated material: one was coated with nitrocellulose and the other was untreated. A 2 μL volume of a 1 mM angiotensin II in 1:1 (v/v) methanol and 0.1% aqueous TFA was deposited on a microscope slide. The slide was mounted 450 μm from either the nitrocellulose coated or uncoated target microscope slide. A single spot on the sample slide was irradiated with 300 shots of 0.5 mJ of focused laser energy at 2.94 μm laser wavelength. After laser ablation sample transfer, CCA matrix was sprayed on both of the nitrocellulose coated and uncoated target slides to which the peptide had been transferred and both slides were analyzed with MALDI mass spectrometry.

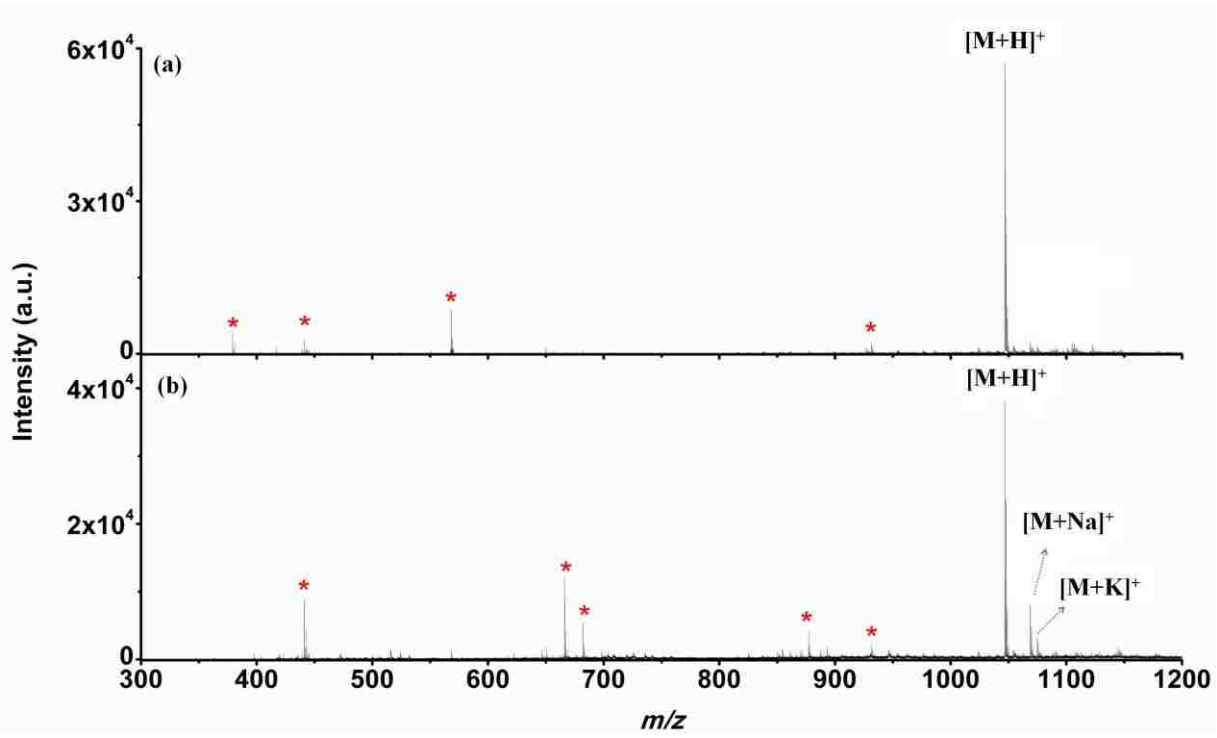

Figure 6-2. MALDI mass spectra of angiotensin II with infrared laser ablation sample transfer to a conductive slide coated (a) with nitrocellulose and (b) without nitrocellulose. Matrix peaks are indicated with asterisks.

Figure 6-2a shows the mass spectrum obtained from the nitrocellulose-coated target slide, and Figure 6-2b shows the mass spectrum obtained from the uncoated target slide. The spectra contain matrix ions (indicated with asterisks), the protonated peptide molecule, $[M + H]^+$, and alkali adduct ions. The abundance of the peptide $[M + H]^+$ ion, whether obtained from peak height or peak area, is greater with nitrocellulose. Additionally, the alkali adduct peaks are less intense. This may result from the nitrocellulose binding alkali metal impurities, thereby reducing the abundance of peptide adduct ions, resulting in enhancement of the $[M + H]^+$ ion signal.[35]

To test the utility of laser ablation sample transfer for MALDI imaging, the IR laser was used to transfer a patterned deposition of peptide sample from one microscope slide to another,

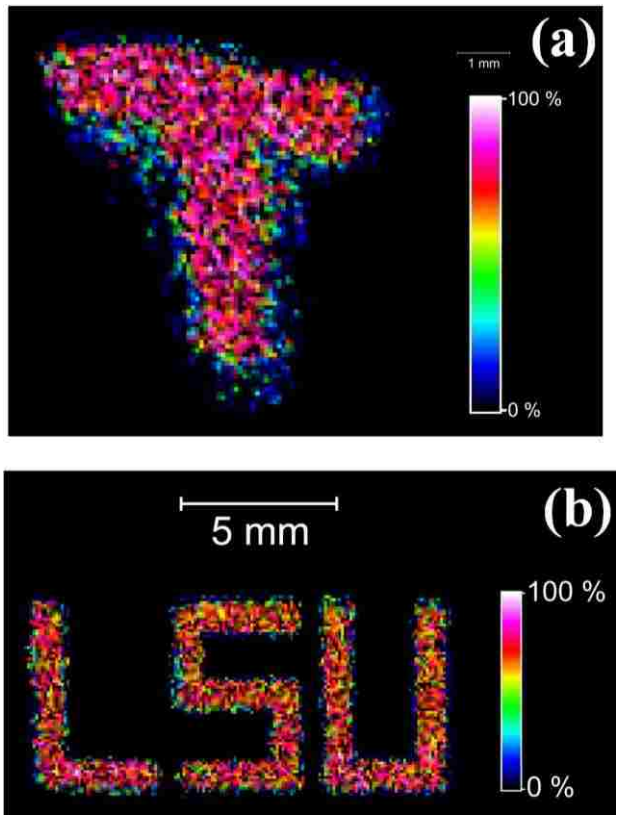

Figure 6-3. MALDI image of peptide spots transferred by IR laser ablation (a) at the indicated laser fluences (at a spacing of 450 μm) and (b) at the indicated slide-to-slide distances between a sample slide and a MALDI target (at a laser fluence of 3 kJ/m^2).

after which the receiving microscope slide was subjected to MALDI imaging. A "T" shaped deposit was created on a microscope slide using a small stencil and TLC sprayer. The character was approximately 5 mm square comprising 1.3 mm lines. The stenciled slide was placed against a nitrocellulose slide with a 450 µm gap. The two slides were mounted on the xy translation stage and translated under the IR laser at 300 µJ pulse energy. A 10 × 10 mm square was irradiated at 30 µm/s with 20 µm spacing between scan lines. After the peptide was transferred, a CCA matrix solution was sprayed onto the slide which was then subjected to MALDI imaging. Figure 6-3a shows he image of protonated angiotensin II ion (*m/z* 1047). The spatial distribution of the peptide matches the T stencil with small distortion that may be attributable to the alignment of the stencil above the nitrocellulose-coated slide or the radial expansion of the ablated material. Selective sample transfer can also be used to transfer biological molecules with spatial selectivity. Figure 6-3b shows a MALDI image of the block letters "LSU" that was created by selectively irradiating a thin film of angiotensin II peptide and transferring it to a nitrocellulose covered microscope slide at 70 µm distance and 0.3 J/cm^2 laser fluence. As

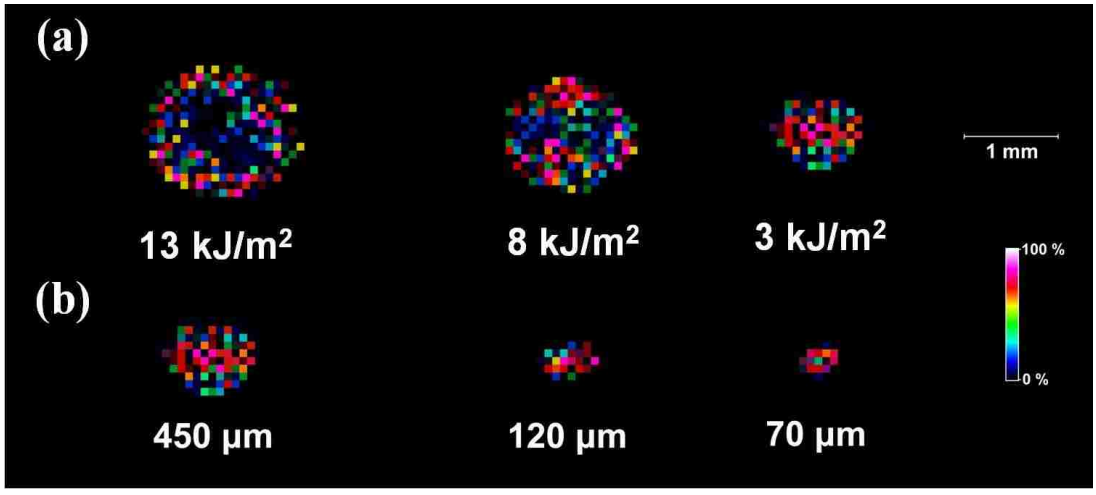

Figure 6-4. MALDI image of peptide spots transferred by IR laser ablation (a) at the indicated laser fluences (at a spacing of 450 µm) and (b) at the indicated slide-to-slide distances between a sample slide and a MALDI target (at a laser fluence of 3 kJ/m^2).

indicated above, glycerol was used to assist the IR laser ablation. The segments of the block letters were scanned 3 times at a spacing of 600 μm. After the peptide was transferred to the MALDI target, a CCA matrix solution was sprayed onto the MALDI target. A 18 × 9 mm rectangular region of the MALDI target was then scanned to obtain the MALDI image.

In order to gauge the dispersion of material upon laser ablation, the laser energy and slide spacing were varied and images of peptide transfer were obtained. For this experiment, a 1 mM solution of angiotensin II was sprayed on a sample target. After air-drying, a glycerol solution was sprayed over the sample slide to assist the IR laser ablation. The slide was irradiated at a single spot at laser fluences of 3, 8, and 13 kJ/m^2 at a distance of 450 μm and at distances of 70, 120, and 450 μm using 200 shots at 20 Hz repetition rate. After transfer of material from the sample slide, the target slide was spray coated with CCA matrix and imaged using MALDI. The transferred spots are shown in Figure 6-4 for different IR laser Figure 6-4b). The spot size of the transferred material on the MALDI target is much larger than the IR laser spot size at high laser fluence (Figure 6-4a) and greater distance (Figure 6-4b). In addition, at higher IR laser fluences (8 and 13 kJ/m^2), a donut shaped image is observed. This could be due to ablation of the transferred sample or due to hydrodynamic ejection of material, fluences (Figure 6-4a) and different distances of the sample target from the MALDI target (which can result in removal of material from the sides of the ablation crater.[36] The size of the spot of transferred material increases with greater spacing between the slides due to the radial dispersion of the plume of ablated material. The increase in spot size with spacing in Figure 6-4b corresponds to an expansion angle of 110°, similar to that observed for the IR ablation of glycerol at 2.94 μm.[36]

As a further test of the spatial resolution achievable using laser ablation sample transfer, a set of lines of peptide was transferred and imaged. For this experiment, a 1 mM solution of

angiotensin II was sprayed on a microscope slide. After air drying, a glycerol solution was sprayed over the slide. The slide was placed on top of the target with a spacing of 70 μm and irradiated with a series of single lines at 3 kJ/m^2 laser fluence. The gaps between laser ablated lines were 1 mm, 800 μm, 600 μm, 400 μm, and 300 μm. Figure 6-5 shows a MALDI image of

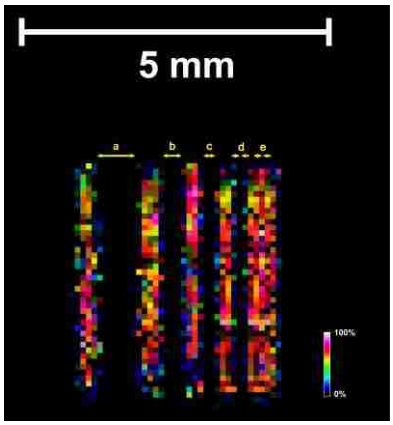

Figure 6-5. MALDI image of a MALDI target onto which angiotensin II peptide was transferred using an IR laser to ablate parallel lines with gaps of (a) 1 mm, (b) 800 μm, (c) 600 μm, (d) 400 μm, and (e) 300 μm.

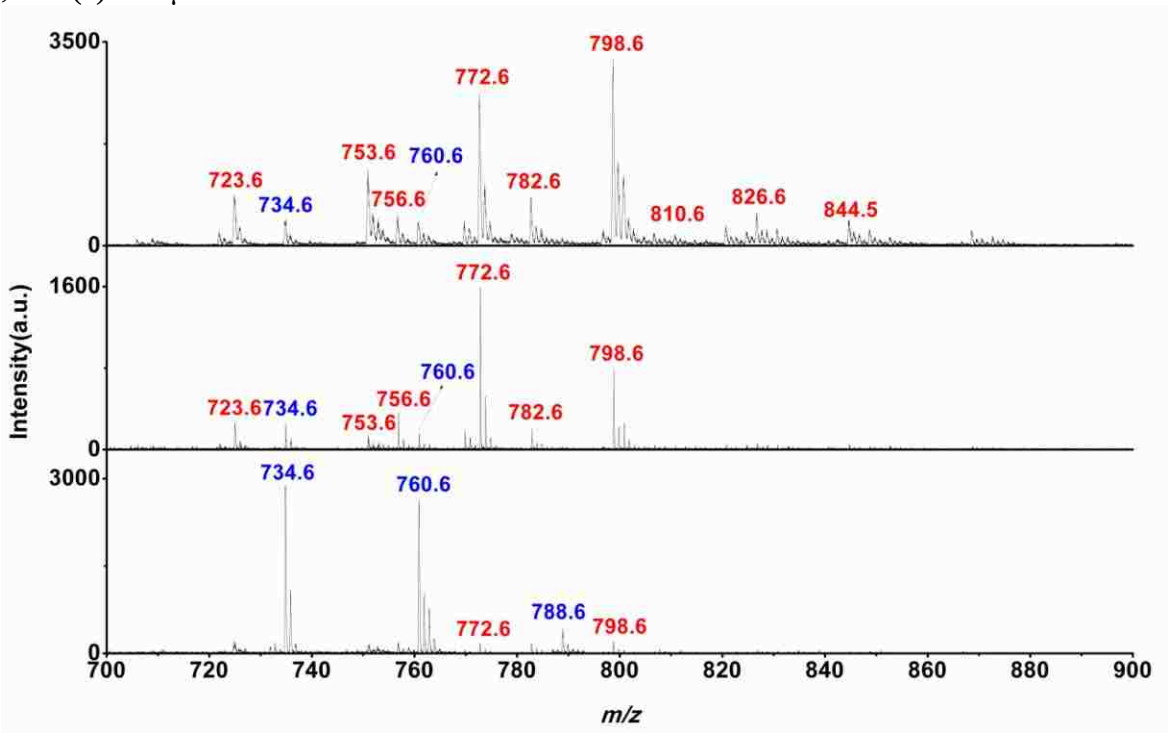

Figure 6-6. MALDI mass spectra of mouse brain tissue obtained by (a) standard MALDI, (b) IR laser transfer to a matrix film, and (c) IR laser transfer with subsequent matrix addition.

the transferred lines on the target obtained with the different spacing. From this image, it can be seen that the minimum distinguishable spacing of the transferred lines on the target is 400 μm. This is well above the diffraction limit and could potentially be improved using a shorter focal length lens[32] or near field optics.[8]

Mouse brain tissue was cut into 10 μm thick sections for MALDI imaging and for laser ablation sample transfer. Two laser ablation transfers were performed: one tissue section was ablated onto a nitrocellulose-coated slide with matrix spray coated later and the other ablated onto a slide coated with both nitrocellulose and matrix. A conventional MALDI image was obtained from a section that was prepared by spraying the matrix directly onto the tissue. Mass spectra obtained from these preparations are shown in Figure 6-6. The slide spacing was 70 μm, and the IR laser fluence was 3 kJ/m^2.

Figure 6-6a shows mass spectrum obtained from the matrix coated tissue section in the m/z range between 700 and 900 where phospholipids are typically detected.39 In this spectrum, prominent ions at m/z 734.6 $[PC(32:0) + H]^+$, 756.6 $[PC(32:0) + Na]^+$, 772.6 $[PC(32:0) + K]^+$, 760.6 $[PC(34:1) + H]^+$, 782.6 $[PC(34:1) + Na]^+$, 798.6 $[PC(34:1) + K]^+$, 788.6 $[PC(36:1) + H]^+$, 810.6 $[PC(36:1) + Na]^+$, and 826.6 $[PC(36:1) + K]^+$ are shown.[13,37-39] Here, PC indicates phosphatidylcholine, and the numbers in parentheses indicate the alkyl chain length and number of double bonds, respectively. The mass spectra resulting from laser ablation sample transfer are comparable in intensity to the direct MALDI analysis: the signal from the matrix precoated slide is approximately half as intense (Figure 6-6b), and the post-transfer matrix addition yields more than 80% of the direct MALDI signal (Figure 6-6c). There are also fewer alkali metal adducts for the sample in which the matrix was added after the sample transfer. This is possibly a result of the partial solvation of the alkali ions and their removal through interaction with nitrocellulose.

The removal of salt impurities by a nitrocellulose comatrix is a phenomenon that has been demonstrated previously.[35]

Laser ablation sample transfer can be used to concentrate material from a large area of the tissue onto a smaller region of the MALDI target. Here, the tissue slide was moved under the laser, and the target was held in place to capture the material on sections (Figure 6-7a, b, c, respectively). The sections were irradiated at a slide to target spacing of 100 μm and a fluence of 3 kJ/m^2. After transfer, 2 μL of DHB matrix was added to the target that was using MALDI in reflectron mode. The spectra contain intense peaks corresponding to phospholipids in the *m/z* 700-900 range and peaks corresponding to gangliosides in the *m/z* 1400−1600 range.[40,41] The MS signal intensity increases with the irradiated area in a roughly linear fashion.

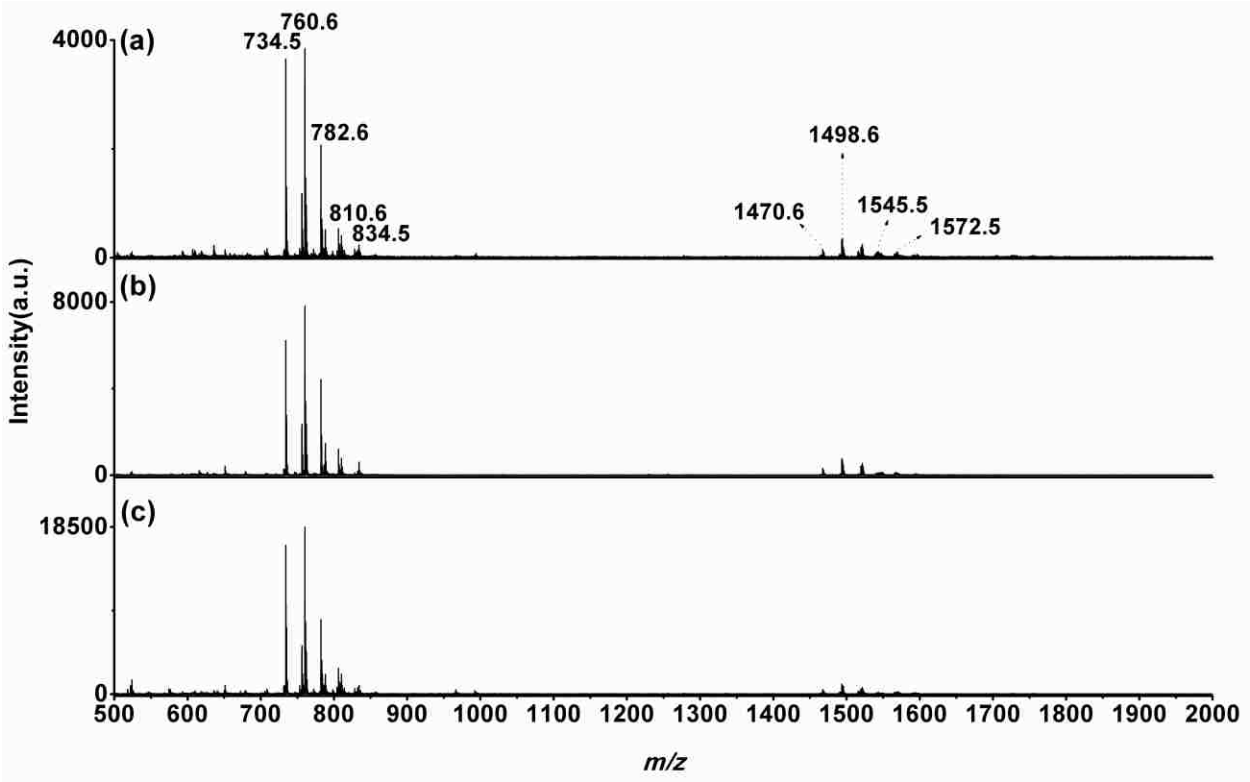

Figure 6-7. MALDI mass spectra obtained by area-to-spot concentration irradiating areas of (a) 0.3, (b) 0.9, and (c) 1.2 mm^2 of mouse brain tissue sections and collecting on a single spot.

MALDI images of the mouse brain tissue prepared as tissue sections or by laser ablation sample transfer are shown in Figure 6-8. The sample preparation and transfer was identical to that indicated above for Figure 6-6, and the prominent phosphatidylcholine peaks were used to

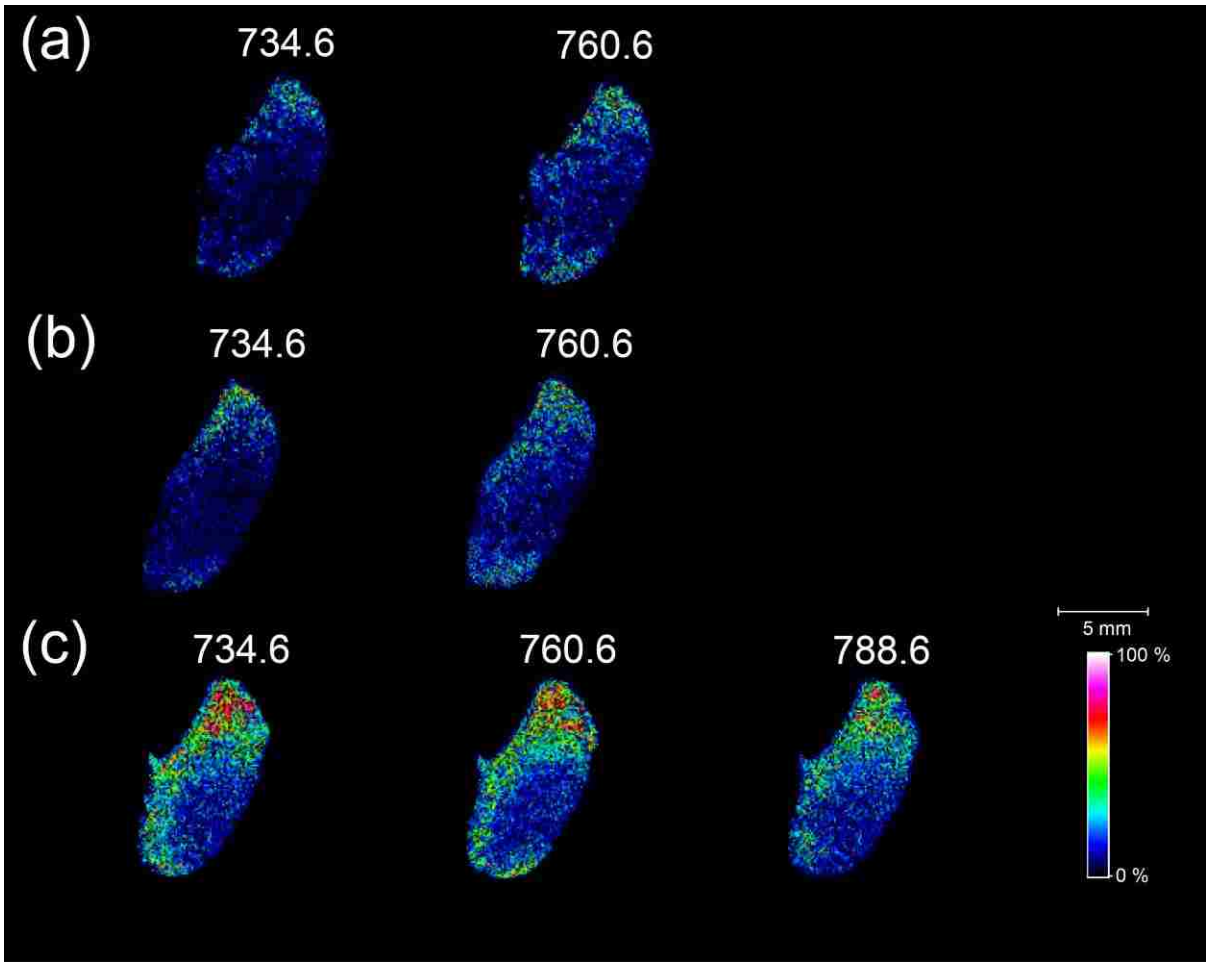

Figure 6-8. MALDI images of mouse brain sections (a) standard MALDI, (b) IR laser transfer to a matrix film, and (c) IR laser transfer with subsequent matrix addition.

generate the images. The spatial distribution from the mouse brain tissue with laser ablation sample preparation, both with the matrix pre-coated slide and post-transfer coating with matrix, is similar to that of the standard MALDI analysis.

Because the ablation sample transfer does not consume all of the tissue material, it is possible to transfer to multiple targets for different sample preparations. To demonstrate this

capability, laser ablation sample transfer from single mouse brain tissue sections was used to deposit material onto multiple targets. Figure 6-9 shows mass spectra and images from multiple sample transfers. Figure 6-9a is the transfer onto a NALDI target, and Figure 6-9b is a second transfer to another NALDI target from the same tissue section. The sample transfer used a slide spacing of 70 μm, and IR laser fluence was 3 kJ/m^2. A 10 Hz laser repetition rate and 30 μm/s translation stage velocity was used. The spectra and images were obtained with no treatment of the NALDI target following the transfer. The signal was reduced by a factor of 10 on the second laser pass. Figure 6-9c was obtained from second sample transfer to a target slide coated with SA after an initial sample transfer to a target slide coated with DHB. The signal was reduced by a factor of 10 for the second laser ablation transfer MALDI run as well.

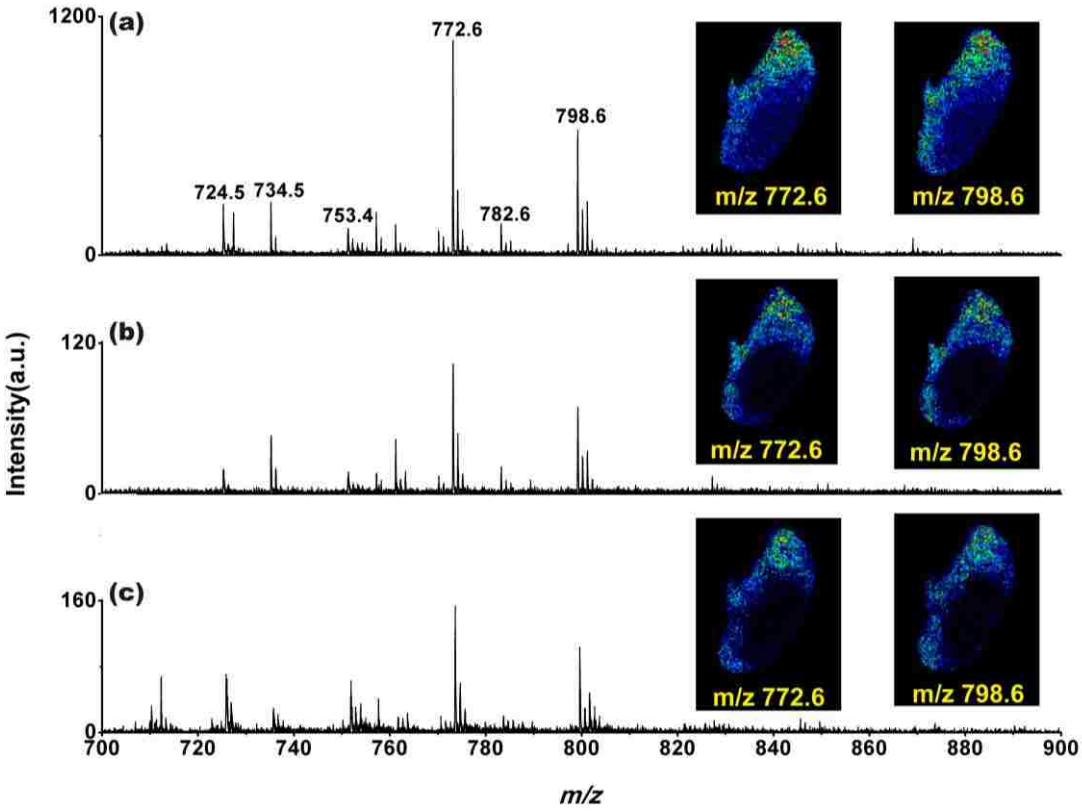

Figure 6-9. MALDI spectra and representative images from multiple IR laser sample transfers: (a) first transfer onto a NALDI target, (b) second transfer to a NALDI target from the same tissue section, and (c) second sample transfer to MALDI slide coated with SA matrix.

6.4 Summary

Ambient sampling with laser ablation sample transfer was demonstrated for MALDI imaging using a commercial TOF mass spectrometer. With a mid-infrared laser operating in the 2.94 μm wavelength region, it was possible to ablate material from a tissue section and transfer it to a microscope slide for MALDI imaging. Due to the expanding plume, the size of the transferred material increased as the distance between the two targets increased. The expanded distribution of the ablated material limited the spatial resolution to approximately 400 μm. Additionally, high energies were found to produce an uneven distribution of transferred material. The spatial distribution phospholipids from mouse brain tissue prepared by laser ablation sample transfer were similar to that of the standard MALDI imaging. Concentration of ablated material from area to spot was demonstrated, and the increase in signal was approximately linear with the area ablated. Ablation deposition onto multiple analysis targets was demonstrated with ablation onto targets coated with different matrix from the same tissue sample. Direct ablation onto nanostructured (NALDI) targets was demonstrated, eliminating the need for either ultrathin tissue sections or tissue blotting. Ongoing work is directed at improving the spatial resolution of material transfer using improved optics.

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CHAPTER 7. CONCLUSIONS AND FUTURE DIRECTIONS

Ambient mass spectrometry can be used to analyze samples in their native environment with little or no sample preparation. Due to its capabilities, ambient MS has been attractive for biological analysis, including tissue imaging. However, the direct analysis of a biological sample makes measurement of relative low abundant species in the sample difficult. In this research, I have developed a new ambient sampling technique for mass spectrometry that can be coupled both with separation techniques and imaging for biological sample analysis. The approach used in this project was based on IR laser ablation sample transfer to a solvent or a surface. An IR OPO system operating at 2.94 μm wavelength was focused onto samples for ablation at atmospheric pressure. The ablated materials were transferred into the solvent or the surfaces and analyzed with ESI and MALDI in either off-line or on-line mode.

A laser ablation sample transfer for off-line ESI and MALDI using a static sampling solvent collection system was demonstrated in Chapter 3. With the collection system, the materials ablated by the IR laser were captured in a solvent droplet, and the solvent was analyzed both by MALDI and ESI. The efficiency of material capture in the droplet is estimated to be 1% with approximately 10 pmol estimated to be transferred to the droplet in a typical experiment. More than 1 mJ IR laser energy could be used for laser ablation sample transfer without sample degradation observed for either peptides or proteins observed with either electrospray or MALDI. The IR laser ablation sample transfer was used to analyze complex biological mixtures for MALDI and ESI analysis.

The laser ablation sample transfer for on-line ESI and on-line LC/ESI using a flowing solvent collection system was demonstrated in Chapter 4, using peptide and protein standard

solutions both with direct analysis and with post-ablation separation. The IR laser operating in the 2.94 μm wavelength region was used to ablate material from sample and transfer it to a flowing solvent stream. A peptide and protein mixture was ablated and separated with LC. The efficiency of material transfer from the target to the liquid flow is approximately 2%. With this system, the ablated peptide and protein mixtures were captured, injected, and each peptide and protein was resolved.

In Chapter 5, laser ablation sample transfer for on-line CE/ESI was presented. The flowing solvent collection system for on-line ESI was modified to load the captured materials in a flowing solvent into the CE capillary by electrokinetic injection. With the system, the number of theoretical plates for the peptides ranged from 1100 to 2900 and resolution between 1 and 2 was obtained. The quantity of material transferred from dried spots to the CE system was 15 fmol for bradykinin, 20 for neurotensin, 40 for angiotensin II and 10 for leucine enkephalin. Reproducibilities (n=4) for migration times were 1.4% for bradykinin, 2.3% for neurotensin, 2.9% for angiotensin II and 1.1% for leucine encephalin.

In Chapter 6, samples on a sample slide were ablated in transmission geometry and captured on target slides for MALDI imaging. The expanded distribution of the ablated material limited the spatial resolution to approximately 400 μm. The spatial distribution of phospholipids from mouse brain tissue prepared by laser ablation sample transfer was similar to that of standard MALDI imaging. Concentration of ablated material from area to spot was demonstrated, and the increase in signal was approximately linear with the area ablated. Ablation deposition onto multiple analysis targets was demonstrated with ablation onto targets coated with different matrices from the same tissue sample. Direct ablation onto nanostructured (NALDI) targets was demonstrated, eliminating the need for either ultrathin tissue sections or tissue blotting.

One of the future directions will focus on improving the spatial resolution of material transfer using a near-field laser ablation technique. In this technique, a laser ablates materials in the near-field by using a tapered optical fiber with an aperture smaller than the wavelength of light or a thin silver needle with a tip radius in the hundreds of nm range tip which is kept within several tens of nm distance above the sample surface to overcome the theoretical diffraction limit which limits high-resolution laser ablation MS. The near-field laser ablation technique has been demonstrated with electron ionization, inductively coupled plasma ionization, and laser desorption ionization of atoms and small molecules.[1-6] However, near-field spatial resolution with peptide and protein mass range remains a challenge. Laser ablation sample transfer to a solvent followed analysis using an ESI and MALDI will allow for the analysis of peptides and proteins in a single cell. In this approach, the ablated material is captured in a nanoelectrospray capillary tip with a 1 μm inner diameter. The tip is then mounted in the source of a commercial mass spectrometer and ions are formed by static nanoelectrospray ionization. Alternatively, the ablated material will be captured on a needle surface and directly analyzed it by MALDI.

A second future direction will focus on designing an interface for coupling microfluidic chips with the laser ablation sampling technique for high throughput biochemical analysis. The analysis of proteins generally requires many sample preparation steps such as extraction, digestion, and separation that is time consuming and labor-intensive. In recent years, a microfluidic chip has been reported for automating continuous-flow protein sample preparation.[7] The sample preparation includes desalting, preconcentration, digestion, and separation prior to ESI/MS analysis.[8-10] However, the approach requires sample collection steps before loading the sample into the microfluidic chip. Laser ablation sampling with droplet capture will provide a new method for spatially resolved sampling into a microfluidic device. The combination of laser

ablation sampling with the microfluidic chip will provide further automation capabilities and process integration into the sample processing pipeline.

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Sung-Gun Park was born in Changwon, Republic of Korea. He graduated with a Bachelor of Science degree from the department of chemistry, Changwon National University, Republic of Korea, in 2003. Following his graduation he extended his study in analytical chemistry with his advisor Dr. Yong-Ill Lee and received a Master of Science degree in 2005. During his graduate study, his research project studied the interaction of transition metals with small peptide and the interaction of small peptide with pesticides using electrospray ionization tandem mass spectrometry (ESI-MS/MS). After receiving his master degree, he was employed at Changwon National University as a teaching assistant. After almost two years of employment experiences, he had decided to extend his study in USA. At Western Carolina University (WCU), he studied the relationship between heavy metals and peptides using ESI-MS/MS with his advisor Dr. David J. Butcher in 2009 for his second master degree in chemistry. In 2010, he joined Dr. Kermit K. Murray research group at Louisiana State University (LSU) for the doctoral program of analytical chemistry. During his doctoral program, the main focus of his research is the development of laser ablation sampling technique that use an infrared laser to ablate material that is captured in a solvent or a surface for electrospray ionization or matrix assisted laser desorption/ionization. This approach also allows subsequent analysis by separation methods such as liquid chromatography or capillary electrophoresis, either on line or off line. Ambient laser-based mass spectrometry imaging incorporating separations allows both spatially resolved analysis of minor sample components and significantly enhances the accessible dynamic range. He is currently a member of the American Chemical Society (ACS) and the American Society for Mass Spectrometry (ASMS). He has presented his research at many national conferences and he has four publications and one manuscript in progress as a first author. He is currently a

candidate for the degree of Doctor of Philosophy in chemistry, which will be awarded at the December 2013 commencement.