MULTIVARIATE DATA ANALYSIS STRATEGIES FOR BIOLOGICAL APPLICATIONS OF MATRIX-ASSISTED LASER DESORPTION/IONIZATION MASS SPECTROMETRIC IMAGING

By

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To my friends and family
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Matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI MSI) is a label-free imaging technique utilized to determine the spatial distribution of biomolecules in tissue. Although MALDI MSI is a proven technique for targeted analysis, global analyses such as those common in the “omics” platforms have largely eluded researchers. Initially, the difficulty in performing global analyses stemmed from instrumentation; however, modern instrumentation allows for the simultaneous detection of thousands to millions of analytes from a single interrogated position. Although datasets that contain approximately 2,000,000 mass features can be collected quite rapidly (~1–10 hours for a 1 cm² sample), processing this dataset from a univariate perspective takes orders of magnitude longer. Thus, data processing is currently the major hindrance to performing global MSI analyses.

Multivariate data analysis techniques commonly practiced in the “omics” platforms (e.g., LC-MS) provide efficient methods to reduce the dimensionality of complex datasets. One such technique, principal component analysis (PCA), performs data reduction by determining the axes of greatest variance within a dataset. The result is
that in the ideal scenario, variability between a case and control, or different regions of
tissue for MSI, can be differentiated using a single principal component. Following
rotation of the multivariate dataset, the original samples are projected onto principal
component axes, and the separation of samples in principal component space can be
visualized using a 2- or 3-dimensional scores plot. The mathematical formula for the
principal component axes is a summation of the original variables multiplied by their
respective weighting factors, or loadings. Thus, the loadings can be inspected to
determine a variable’s contribution to the separation in principal component space.

This dissertation describes PCA methodologies that strive to reduce analysis time,
while simultaneously extracting the most relevant information from an MSI dataset.
Following a brief introduction to MSI and multivariate data analysis, the developed
methodologies are utilized for three biological applications: 1) positive mode MSI
analysis of a model system for myocardial infarction, 2) positive and negative mode MSI
analysis of myocardial infarction using 9-aminoacridine as a MALDI matrix, and 3) the
identification of biomolecules from Caenorhabditis elegans and related species.
Mass Spectrometric Imaging

Concept

Mass spectrometric imaging (MSI) is an analytical technique utilized to generate chemically selective images by directly interrogating a sample surface.\(^1\) To date, two imaging modalities exist: microprobe mode imaging and microscope mode imaging. Despite the imaging modality, the general concept remains the same. In brief, an ionization source is utilized to generate ions from a discrete position (or area) on a sample surface. The ions are then transported to a mass analyzer for immediate detection, or alternatively, isolation, fragmentation, and subsequent detection. The resulting mass spectrum is stored and a relative X,Y position is associated with the mass spectrum. Following detection, an X,Y stage is utilized to move the sample by a specified raster step size, and the process is repeated until the entire tissue has been interrogated. An image can then be generated by extracting the ion intensity for a specified mass-to-charge ratio \((m/z)\), and plotting the intensity versus the X,Y position on the sample.

Microprobe and microscope mode imaging

As previously stated, two modalities exist for MSI. The most common imaging modality is microprobe mode imaging, wherein an ionization source is utilized to interrogate a relatively small area of the target sample.\(^2\) The generated ions are then mass analyzed, and all of the ions are treated as being from the same position on the target sample. Following collection of the first mass spectrum, the sample is rastered with respect to the stationary ionization source, and the ionization and mass analysis
process is repeated. In contrast, microscope mode imaging utilizes an ionization source to interrogate a larger area of the target sample. As before, the ions are transferred to the mass analyzer following ionization; however, in this modality, the spatial orientation of the ions is maintained from ionization to the detector for mass analysis.²

Both modalities have a number of inherent advantages and pitfalls. First, in microprobe techniques, the spatial resolution of the imaging experiment is often limited by the ionization source. With microscope techniques, the spatial resolution is often limited by the ion optics and the resolution of the position-detector. Although better spatial resolution can be achieved with microscope techniques, the instrument cost and sensitivity may suffer. Thus, for the purposes of this dissertation, all MSI from this point forward will refer to microprobe mode imaging.

**Ionization sources**

The initial step in the MSI process is that of ionization, wherein neutral molecules from the sample surface are ionized and subsequently transferred to the mass analyzer. A number of ionization sources are currently commercially available with imaging capable mass spectrometers. Although choice of a suitable ionization source is largely dependent upon the sample and analytes to be detected, the three most common ionization sources for MSI are matrix-assisted laser desorption/ionization (MALDI), secondary ion mass spectrometry (SIMS), and desorption electrospray ionization (DESI). Each of these ionization sources present unique advantages, and will be discussed further below.

**MALDI**

Matrix-assisted laser desorption/ionization (MALDI), first reported by Karas et al.³,⁴ and Tanaka et al.,⁵ is currently the most common ionization source utilized in MSI.
MALDI is a ‘soft’ ionization technique (i.e., producing a relatively low amount of source fragmentation) that utilizes a UV-laser to induce both desorption and ionization of analytes. The most common lasers utilized for UV-MALDI are a N\textsubscript{2} gas laser (\(\lambda=337\) nm) or a frequency-tripled Nd:YAG solid-state laser (\(\lambda=355\)). Typically, N\textsubscript{2} lasers offer pulse rates between 10 and 100 Hz, whereas Nd:YAG lasers offer pulse rates around 1 kHz. This pulse rate often dictates the mass analyzer utilized with the respective MALDI source. For instance, N\textsubscript{2} lasers are often paired with relatively slow mass analyzers (e.g., ion traps), whereas Nd:YAG lasers are often paired with time-of-flight (ToF) mass analyzers. Gas lasers, including a N\textsubscript{2} laser, are also relatively inexpensive compared to solid-state lasers;\(^6\) thus, when the repetition rate is not needed to accelerate experimentation, gas lasers are often chosen.

The target sample is coated with a UV-absorbing matrix to aid in both desorption and ionization of the analytes from the sample surface. Although there are various MALDI matrices commercially available, most are small organic acids (for positive mode) or bases (for negative mode) that absorb strongly in the UV. Choice of a MALDI matrix is largely dependent upon the analyte of interest. For instance, sinapinic acid is commonly utilized for large intact proteins, whereas \(\alpha\)-cyano-4-hydroxycinnamic acid is commonly utilized for smaller peptides.\(^7\) The matrix is typically saturated in a solvent system that can readily extract analytes of interest from the target. Upon evaporation of the matrix solvent, matrix and analyte cocrystals are formed. Irradiation of these cocrystals with a UV-laser generates singly charged ions over a wide \(m/z\) range (Figure 1-1).
The ionization mechanism for MALDI is still widely debated throughout the MS community; however, two models have been proposed: the lucky survivor model\textsuperscript{8} and the gas-phase protonation model.\textsuperscript{9} The lucky survivor model proposes that analytes are incorporated into the matrix and analyte cocrystals as charged species. Upon desorption, most of the highly charged matrix/analyte clusters become neutralized or undergo charge reduction by counter ions of opposite charge present in the plume; however, a small portion of singly charged ‘lucky survivors’ remain charged.\textsuperscript{8} This mechanism was further refined later to also account for anion formation, in addition to cation formation.\textsuperscript{10} Alternately, the gas-phase protonation model suggests that analytes are not present as charged species in the cocrystals. Instead, matrix radical cations are proposed to be generated following multiphoton ionization. Following formation of the matrix radical cation, a proton can be efficiently transferred to form a protonated matrix ion. Subsequently, transfer of a proton from the protonated matrix ion to the neutral analyte molecule will be dependent upon the proton affinities for both species, and is thought to be an thermodynamically favorable process in many cases.\textsuperscript{9} Recently, there has even been some evidence for a unified theory that combines both the lucky survivor and gas-phase protonation models.\textsuperscript{11}

Regardless of the ionization mechanism in MALDI, the matrix application process greatly influences the quality of spectra obtained. In general, the ideal application process will maximize the extraction of analytes from the sample, while minimizing the lateral diffusion of those analytes for MSI. The most conventional application technique utilized for coating is pneumatic spraying, including the use of nebulizers and TLC sprayers.\textsuperscript{12} Pneumatic spraying provides a rapid method that produces relatively small
crystal sizes; however, the majority of devices utilized for delivering matrix in this manner are manually controlled, possibly introducing inhomogeneity during coating. A number of commercially available matrix coating apparatuses have been developed to attempt to reduce inhomogeneity, including the Bruker ImagePrep (pneumatic spraying) and the Shimadzu Chemical Inkjet Printer, or ChIP, (microspotting). Although fully automated, these systems prove to be rather expensive. A promising compromise between cost, automation, and crystal size appears to be the use of commercial office inkjet printing technologies for coating MALDI matrix. A modified inkjet printer developed in the Yost lab produced smaller crystal sizes and a more homogenous matrix coating than user-controlled pneumatic spraying devices. Additionally, the price of a standard inkjet printer is inexpensive relative to the ImagePrep or the ChIP. The biggest limitation of commercial inkjet printers appears to be solvent compatibility; standard inkjet printers currently utilize plastic cartridges and print heads that are incompatible with certain solvents (e.g., chloroform). The most recent matrix application technique to gain interest in the MSI community is sublimation. This technique produces a smaller crystal size relative to pneumatic spraying at the cost of reduced time for matrix-analyte interaction and analyte extraction.

**SIMS**

Secondary ion mass spectrometry, or SIMS, is the oldest ionization technique commonly utilized in MSI. In SIMS, reagent ions serving as a focused primary ion beam are directed at a sample surface. The primary ions serve to eject secondary ions from the sample surface for subsequent detection by the mass analyzer. Generation of the primary ion beam can be performed with a number of sources, including liquid metal ion guns (e.g., Ga⁺, In⁺, or Au⁺), solid-state guns (e.g., Cs⁺), or C₆₀⁺ cluster sources.
Depending upon the ion dose delivered to the surface, SIMS can be performed in either static or dynamic modes. For the purposes of this discussion, static SIMS can be considered ion doses lower than $10^{12}$ ions/cm$^2$. When operated in this mode, the primary ions do not penetrate farther than the outermost monolayer of the sample surface. In contrast, dynamic SIMS uses a relatively high ion dose resulting in ejection of the secondary ions further than the outermost monolayer.

Relative to MALDI, SIMS is considered a harsh ionization technique. The relatively high energy interaction between the primary ion beam and the sample surface induces in a large degree of source fragmentation, resulting in reduced selectivity. To illustrate this point, diverse compound classes such as phosphatidylcholine lipid species are fragmented to one or two fragment ions related to the common headgroup, and yield little to no information regarding the intact molecular weight (MW) of the species. Furthermore, under typical operating conditions, the practical upper mass limit for SIMS is $\sim m/z$ 1000. The addition of a matrix molecule to the surface (similar to MALDI) can serve to reduce the amount of source fragmentation, thereby increasing the effective upper mass limit of the technique.

Despite the extensive source fragmentation and relatively low mass limit, SIMS offers a number of advantages over MALDI. Perhaps the most significant advantage is enhanced spatial resolution. In theory, the spatial resolution in MALDI is diffraction limited; however, practical considerations with the optical configuration usually limit MALDI sources to a spatial resolution of $\sim 10$ μm. In SIMS, the practical spatial resolution is limited by the focusing of the primary ion beam and the lateral interaction of the primary ion beam with the surface; modeling has suggested that molecules are
desorbed 5–10 nm from primary ion beam. Currently, practical spatial resolutions in SIMS are routinely reported as being less than 50 nm.

**DESI**

Desorption electrospray ionization (DESI) is an ambient condition ionization technique for MSI that was first reported by Graham Cooks’ group in 2004. In this technique, an electrospray needle is utilized to spray charged droplets at a sample surface. Upon striking the surface, these charged droplets cause simultaneous desorption and ionization of analyte molecules from the sample surface. Interestingly, the ionization mechanism of DESI appears to vary depending on the analyte. Certain analytes (e.g., peptides and proteins) yield multiply charged ions, suggesting a solution-phase ionization mechanism similar to electrospray ionization (ESI), whereas others yield singly charged ions, suggesting a charge transfer ionization mechanism similar to atmospheric pressure chemical ionization (APCI).

Relative to the other traditional MSI ionization techniques, DESI appears to be the most amenable for ambient sampling. There is no need to place the sample under any kind of vacuum, and there is little to no sample preparation (excluding sectioning and mounting) that need be performed. Despite these advantages, DESI demonstrates a spatial resolution of approximately 200 μm, thereby limiting DESI-MSI applications.

**Emerging ionization sources**

Recently, a number of ionization sources have been reported in the literature that may prove valuable for MSI research. These ionization sources include laserspray ionization (LSI), laser ablation electrospray ionization (LAESI), and the liquid microjunction. Although a detailed description of these sources is beyond the scope of this dissertation, each of the emerging sources will be described briefly.
LSI, first reported by Sarah Trimpin and coworkers in 2010,\textsuperscript{20} utilizes a UV-laser to irradiate the back side of a thin sample mounted on either a UV-transparent cover slip or microscope slide. This configuration is commonly referred to as the transmission geometry, and was used on early microprobe instruments such as the LAMMA-500.\textsuperscript{21} Similar to atmospheric pressure (AP) MALDI,\textsuperscript{22} the mounted sample is coated with a MALDI matrix, and held at atmospheric pressure. If the sample is sufficiently thin, the analyte/matrix cocrystals will be ablated by the laser, and the subsequent ions will be transferred to the mass analyzer by means of a heated capillary and ion optics. Although the only deviation from standard AP MALDI experiments is the transmission geometry, vastly different spectra are produced with LSI. In initial studies, lysozyme, a large protein, was analyzed with 2,5-dihydroxybenzoic acid (DHB) as the MALDI matrix using the transmission geometry and a high laser fluence, producing highly charged ions similar to those obtained with ESI.\textsuperscript{20} In contrast, the same sample analyzed in the typical reflective geometry and a low laser fluence produces predominantly singly charged ions. Later, it was determined that neither the geometry nor the laser fluence are relevant for generating multiply charged ions.\textsuperscript{23} Instead, it was determined that a minimal or zero potential difference between the target and the sample inlet and a heated region prior to mass analysis are both required for the observation of these multiply charged ions.\textsuperscript{23} Based on these observations, an ionization mechanism similar to ESI has been proposed, in which charged matrix/analyte clusters are partially desolvated, releasing matrix molecules until a highly charged analyte droplet is obtained.\textsuperscript{23}
Regardless of the ionization mechanism, LSI offers a couple of interesting advantages over traditional ionization sources. First, in the transmission geometry, the optics for the laser can be brought sufficiently close to the back side of the target slide or cover slip. The implication here is that the principles of near-field optics can be applied, potentially allowing the MALDI spot size to approach, or even surpass, the diffraction limit, suggesting the potential for submicron imaging. Second, the ability to produce multiply charged ions permits the use of ion traps for a wider range of analytes. Thus, intact high molecular weight proteins, which normally require digestion prior to analysis on ion trap mass spectrometers, may be brought into the effective m/z range for these mass analyzers.

LAESI combines the use of a laser to ablate analytes from a sample surface and an orthogonal ESI source for postionization. This technique offers a number of interesting advantages similar to LSI. The utilization of postionization by ESI offers the ability to generate multiply-charged ions, permitting the analysis of high-molecular weight species. Furthermore, as a mid-IR laser is often used for ablation, water-containing or ‘wet’ samples can be analyzed without sample preparation other than sectioning.

The final family of sources that merits consideration for MSI is that of the liquid microjunction variety. In these sources, a solvent stream that contacts the surface of a sample serves to simultaneously extract and transport the analytes to the ionization source, typically ESI. Similar to LAESI and LSI, liquid microjunction sources allow for multiple charging of analytes when combined with ESI, making them suitable for pairing
with ion traps. However, the major disadvantage associated with these types of sources is spatial resolution, which is currently on the order of 500 μm.  

**Mass analyzers**

Following ionization, the generated ions are transferred to the mass analyzer by differential pumping and a series of ion optics. At present, a number of mass analyzers are available for integration with sources amenable to MSI. Historically, the most common mass analyzers for MSI have been ion traps and time-of-flight mass analyzers; however, there has been a recent surge of interest in the MSI community in Fourier transform mass analyzers. The operational principles and the relative merits of each of these mass analyzer classes will be discussed in this section.

**Ion traps**

The two most common ion traps used for MSI are the three-dimensional quadrupole ion trap (QIT) and the two-dimensional linear ion trap (LIT). The concept of trapping ions in a quadrupolar field was initially proposed in Wolfgang Paul’s patent describing quadrupole mass filters. Consequently, the operation of a QIT derives from a quadrupole mass filter, in which four parallel hyperbolic (or cylindrical) rod electrodes are utilized in a square array. A combination of radio frequency (RF) and direct-current (DC) potentials is applied to opposite pairs of rods to generate the quadrupolar field. Depending on the magnitude of the potentials applied and the mass-to-charge (m/z) of the ion, the trajectory of the ion will either be stable, passing through the quadrupole mass filter, or unstable, causing the ion to collide with a rod or become ejected from the mass filter.

The stability of an ion within a quadrupolar field is determined by a set of non-linear equations known as the Mathieu equations. As applied to a quadrupole mass
filter, a Mathieu stability diagram can be plotted in terms of the parameters \( a \) and \( q \), of which the formulas for these two parameters are listed below (Equation 1-1 and Equation 1-2). The parameter, \( a \), is influenced by the mass of an analyte \( (m) \), the electronic charge \( (e) \), the radius of an electrode \( (r_0) \), the drive frequency \( (\Omega) \), and the applied DC potential \( (U) \).

\[
a = \frac{8eU}{mr_0^2\Omega^2} \tag{1-1}
\]

In contrast, \( q \) is influenced by the mass, the electronic charge, the radius, the drive frequency, and the applied RF potential \( (V) \).

\[
q = \frac{-4eV}{mr_0^2\Omega^2} \tag{1-2}
\]

The positive portion of the first stability region for a quadrupole mass filter is shown in Figure 1-2, with areas of stability shaded in grey. Mass discrimination in the quadrupole mass filter is achieved using the apex of the stability diagram. If one applies the appropriate RF and DC potentials, an operating line is created where only a small range of mass-to-charge \( (m/z) \) values falls within the uppermost portion of the stability diagram. Furthermore by increasing the DC and RF potentials while maintaining the ratio of DC to RF potential, a mass spectrum can be scanned in order of increasing \( m/z \).

Although many of the fundamentals are similar, the QIT has a much different configuration than a quadrupole mass filter. The QIT has three electrodes as opposed to the traditional four in the quadrupole mass filter. Two of the QIT electrodes have an outer hyperboloidal geometry, serving as end-caps, and the remaining electrode has an inner hyperboloidal geometry, serving as a ring electrode.\(^{27}\) Furthermore, the optimal theoretical radius for the ring electrode is only half that of the distance between the two
end-caps, in contrast to the quadrupole mass filter, in which all four electrodes are equidistant. As a result, the parameters \( a \) and \( q \) for the Mathieu stability diagram must be considered in both the end-cap separation (axial direction) and the ring electrode radius (radial direction). Thus, for a QIT, the parameter \( a \) is now a function of mass \( m \), the electronic charge \( e \), the DC potential \( U \), the radius of the ring electrode \( r_0 \), the distance between the ring electrode and either end-cap \( z_0 \), and the drive frequency \( \Omega \). The equation for \( a \) in the axial direction \((a_z)\) is shown below:

\[
a_z = -\frac{16eU}{m(r_0^2 + 2z_0^2)\Omega^2}
\]

(1-3)

Due to the relative radii between the end caps and ring electrode, \( a \) in the radial direction \((a_r)\) is simply:

\[
a_r = -\frac{a_z}{2}
\]

(1-4)

Similarly, \( q \) in the axial direction \((q_z)\) is defined as:

\[
q_z = \frac{8eV}{m(r_0^2 + 2z_0^2)\Omega^2}
\]

(1-5)

Finally, \( q \) in the radial direction \((q_r)\) is defined as:

\[
q_r = -\frac{q_z}{2}
\]

(1-6)

With an understanding of \( a \) and \( q \) in both the axial and radial directions, the regions of stability can be defined. Formerly symmetrical about the \( q \) axis, the Mathieu stability diagram for a QIT becomes stretched due to the necessity for both radial and axial stability in the ion trap. A typical stability diagram (expressed in terms of \( a_z \) and \( q_z \)) for a QIT is shown in Figure 1-3. Assuming an ion has a stable ion trajectory within an ion trap, the ion will oscillate at a specific fundamental frequency. This fundamental
frequency, known as the secular frequency ($\omega$), is influenced by a secondary trapping parameter ($\beta_u$) and $\Omega$ (Equation 1-7).

$$\omega_u = \frac{1}{2} \beta_u \Omega$$

(1-7)

The Dehmelt approximation defines $\beta_u$ for values of $q$ less than 0.4 and is given as:

$$\beta_u \approx \sqrt{(a_u + \frac{1}{2} q_u^2)}$$

(1-8)

For larger values of $q$, the Dehmelt approximation is no longer an effective approximation for $\beta_u$, and the precise value of $\beta_u$ is found by solving a continuous fraction, which is beyond the scope of this work. Substituting $\beta_u$, and subsequently $a_u$ and $q_u$, into Equation 1-7, one finds that secular frequency is inversely proportional to $m/z$.

Revisiting the stability diagram in Figure 1-3, when 0 DC voltage is applied (i.e., $a_z=0$), the stability diagram intersects with the $q_z$ axis at a value of 0.908, yielding the theoretical low-mass cutoff (LMCO) for a QIT. Consequently, the LMCO is directly proportional to the applied RF voltage, and ions can be selectively ejected in increasing $m/z$ by simply ramping RF voltage. Alternatively ions can be ejected by applying a waveform with the appropriate resonant frequency matching the secular frequency of the ion to be ejected. As we will find later, a combination of these two ejection methods, more commonly known as mass-selective instability, is the most practical approach.

The LIT consists of three hyperbolic quadrupole rod arrays in sequence. The outer two sets of rod arrays are typically shorter in length than the middle set of rods. DC potentials are applied to all three sets of rods to trap the ions within the center section. On the Thermo Scientific LTQ XL, which will be used extensively in the work
of this dissertation, three discrete axial DC potentials are applied. The schematic for the MALDI LTQ XL is shown in Figure 1-4. During ion trapping in positive mode, voltages of −9 V, −12 V, and −7 V are utilized for the front, center, and rear rod arrays, respectively. For negative mode, DC voltages of the same magnitude, but opposite polarity, are applied.

As the design of an LIT closely resembles that of a quadrupole mass filter, Equation 1-1 and Equation 1-2 can be utilized to calculate the parameters $a$ and $q$, respectively. Similarly, the Mathieu stability diagram is the same for an LIT and a quadrupole mass filter; however, it should be noted that during mass analysis, the LTQ XL is operated in RF-only mode (i.e., $a=0$ and only RF voltage is applied), similar to the QIT; thus, the y-axis on the stability diagram can be largely ignored.

Although the operating principles for an LIT are a hybrid of the quadrupole mass filter and the QIT, the LIT offers a variety of practical advantages over the QIT, including increased ion storage volume, ease of ion injection, higher trapping efficiency, and lower mass discrimination. Perhaps the most striking advantage is the increased ion storage volume of the LIT; the practical storage volume of the LIT is dictated by the length of the center section, whereas the practical storage volume of a QIT is dictated by $r_0$. Lengthening $r_0$ in the QIT requires higher RF voltages for efficient trapping; however, axial confinement in the LIT is brought about by DC voltage. This ability to store more ions in the LIT results in reduced space charge effects; the space charge limit can be thought of as the maximum number of ions trapped while maintaining efficient activation, isolation, mass resolution, or mass accuracy. Thus, the more ions...
that can effectively be trapped results in greater sensitivity and increased dynamic range, resulting in a higher performance mass analyzer.

When considered as a whole, ion traps are relatively simple mass analyzers that require little upkeep while providing superior MS\textsuperscript{n} capabilities.\textsuperscript{31} Although stand-alone ion traps are valuable in MSI, these mass analyzers are lacking in performance relative to more complex and expensive mass analyzers, assuming only one stage of mass analysis. The three most prominent disadvantages of ion traps are mass resolution, mass range, and scan speed. Under typical operating conditions, most ion traps demonstrate unit resolution across the usable mass range. Despite the use of resonance ejection voltages and slower scan speeds, this process is highly influenced by space charge effects (discussed above). Furthermore, the mass range of commercial ion traps limits analyses to ions less than 4,500 amu;\textsuperscript{2} however, this upper mass limit has been circumvented in a number of experimental configurations.\textsuperscript{32} Finally, the scan speed of a conventional ion trap is approximately 1 scan/sec. Although this can be improved, an increase of scan speed is often accompanied by a loss in resolution. Thus, this scan speed/resolution tradeoff presents a concerning barrier for rapid high resolution imaging data acquisition. Despite the aforementioned disadvantages, the ease of implementing two or more stages of mass spectrometry (MS\textsuperscript{2} and MS\textsuperscript{n}) should not be overlooked. The importance of this capability for MSI will be highlighted throughout this dissertation.

**Time-of-flight mass analyzers**

Time-of-flight (ToF) mass analyzers are also well-suited for integration with pulsed ionization sources such as MALDI. In MALDI-ToF MS, an ion packet generated by the ionization source is accelerated into a flight tube. Following acceleration, each ion in
the initial ion packet should now have the same kinetic energy. Prior to acceleration, an ion has a potential energy \( E_p \) related to its charge \( (z) \), the charge of an electron \( (e) \), and the applied voltage \( (V) \) (Equation 1-9).

\[
E_p = zeV
\] (1-9)

Following acceleration, the ion has a kinetic energy \( E_k \) that is simply related to the ion’s mass \( (m) \) and its velocity \( (v) \) (Equation 1-10).

\[
E_k = \frac{mv^2}{2}
\] (1-10)

Assuming all of the potential energy is converted to kinetic energy, Equation 1-9 and Equation 1-10 can be equated to form Equation 1-11.

\[
zeV = \frac{mv^2}{2}
\] (1-11)

Thus, if Equation 1-11 is rearranged, we find that the \( m/z \) ratio of an ion is inversely proportional to the square root of its velocity. Furthermore, the time it takes an ion to travel down a drift tube \( (t) \) is solely dependent on the velocity of the ion and the length of the drift tube \( (L) \) (Equation 1-12).

\[
t = \frac{L}{v}
\] (1-12)

By combining Equation 1-11 and Equation 1-12 and solving for time, we find that the drift time of an ion is proportional to the square root of the ion’s \( m/z \) (Equation 1-13).

\[
t = L\sqrt{\frac{m}{2zeV}}
\] (1-13)

Thus, ToF mass analyzers provide a simple method for mass analysis; ions with a greater \( m/z \) ratio travel more slowly down the drift tube and reach the detector later than ions with a lower \( m/z \).
When compared to ion traps, linear ToF mass analyzers exhibit a much higher upper mass limit (~150 kDa). Although linear ToF geometries can analyze higher molecular weight species, the lower mass region of the MALDI MS spectrum (<600 Da) is typically obscured by matrix-related ions. Furthermore, the mass resolution in the linear geometry is fairly poor. The addition of an electrostatic mirror, commonly referred to as the reflectron geometry, compensates for variations in the velocity of isobaric ions, resulting in a much improved mass resolution, far surpassing that of conventional ion traps. Thus, ToFs provide a compromise between mass range and resolution that is well-suited for a number of MSI applications. The major disadvantage for ToF mass analyzers is that they are typically limited to one or two stages of mass analysis when placed in tandem with a quadrupole or second ToF analyzer.

**Fourier transform mass analyzers**

Fourier transform mass analyzers offer an alternative to the destructive methods of ion detection used in other mass analyzers. The conventional process for ion detection involves ions striking a surface (e.g., a conversion dynode or electron multiplier), wherein the collision of the ion with a surface generates a secondary signal that can be measured (e.g., the generation of secondary electrons that are subsequently multiplied and measured as current). In contrast, Fourier transform mass analyzers generate a detectable signal by analyzing the image current generated from trapped ions. Two types of Fourier transform mass analyzers, Fourier transform ion cyclotron resonance and orbitrap mass analyzers, will be discussed in this section.

In Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR MS), ions are confined by a combination of magnetic and electrostatic fields. Confinement is achieved by placing an FT-ICR cell, with applied electrostatic potentials, inside the
central bore of a superconducting magnet. The FT-ICR cell is typically segmented into a number of sections: some of which are utilized for excitation and the remaining sections for detection. Initially, the ions within the trapping area exhibit an incoherent motion.\textsuperscript{33} Thus, following ion injection, the random distribution of ions throughout the ion trap results in a charge balance between the opposing detection plates.\textsuperscript{33} By applying an radio frequency (RF) waveform to the excitation plates that continually increases (or decreases) in frequency, ions of similar \textit{m/z} that are in resonance with the excitation field begin to increase in cyclotron radius within the trapping area, forming coherent ion packets. As the coherent ion packets travel close to the detection plates, the charge of the ion packet induces an image current, which can then be measured. A transient is generated by plotting the magnitude of the image current over time. By performing fast Fourier transform on the transient, the data can be converted from the time domain to the frequency domain, yielding a plot of magnitude vs. cyclotron frequency (\textit{v}_c). Under ideal conditions, the cyclotron frequency exhibits a dependence on the \textit{m/z} of the ion and the magnetic field strength (\textit{B}_0) detailed in Equation 1-14.\textsuperscript{34}

\[
\frac{v_c}{2\pi} = \frac{1.535 \times 10^7 B_0}{m/z}
\]

(1-14)

The major advantage of this manner of detection lies in mass resolution and mass accuracy, offering anywhere between a 10–100-fold improvement over traditional mass analyzers.\textsuperscript{35} Furthermore, the mass resolving power of the mass analyzer is directly proportional to the magnetic field;\textsuperscript{34} thus, as progressively stronger superconducting magnets are fabricated, the resolving power of the technique will continually improve. Currently, there are two major disadvantages to the use of FT-ICR MS instruments: cost and upkeep, both of which stem from the superconducting magnet. Typical high-
performance FT-ICR MS instruments cost at least 1 million USD.\textsuperscript{36} Furthermore, the superconducting magnet must be cryogenically cooled, resulting in the consumption of both liquid helium and nitrogen.

Orbitrap mass analyzers, first introduced by Alexander Makarov in 2000,\textsuperscript{37} provide an alternative to the relatively expensive FT-ICR MS for high-performance mass analysis. The orbitrap consists of two electrodes: an outer “barrel-like” electrode that surrounds an inner “spindle-like” electrode.\textsuperscript{37} In a purely electrostatic field, the position of an ion \((z(t))\) at any given point in time along the axis of the electrodes (i.e., the \(z\) axis) can be thought of as a simple harmonic oscillator, expressed in Equation 1-15.\textsuperscript{37}

\[
    z(t) = z_0 \cos(\omega t) + \sqrt{\frac{2E_z}{k}} \sin(\omega t) 
\]  

(1-15)

In this equation, \(k\) represents the field curvature, \(\omega\) is the frequency of axial oscillation, \(z_0\) is the initial position of the ion, \(t\) is time, and \(E_z\) is the energy characteristic of the ion, as defined by Equation 1-16.\textsuperscript{37}

\[
    qE_z = \frac{(m/2)z_0^2}{k} 
\]  

(1-16)

Under ideal conditions, \(\omega\) also exhibits a dependence upon mass-to-charge \((m/q)\), as expressed in Equation 1-17.\textsuperscript{37}

\[
    \omega = \sqrt{\frac{q}{m}k} 
\]  

(1-17)

Thus, the frequency of oscillation of an ion along the \(z\)-axis is proportional to the ion’s \((m/z)^{-1/2}\)\textsuperscript{.38} Similar to FT-ICR MS, ions of the same \(m/z\) must exhibit coherent motion along the \(z\)-axis for an image current to be generated. Coherence is achieved by rapidly pulsing the ions into the orbitrap at a position offset from the equator, defined as \(z=0\).\textsuperscript{38} Assuming the spread of flight times into the trap is considerably small (on the
order of nanoseconds), the ions will exhibit coherent motion along the z-axis without an excitation waveform. In this fashion, image current is detected by segmenting the outer electrode into two independent sections about the equator. From this point onward, detection and processing proceed parallel to that of FT-ICR MS.

In regards to performance, orbitrap mass analyzers have been reported to have mass resolution and mass accuracy rivaling, or in some cases exceeding, that of FT-ICR mass analyzers. Furthermore, improving the performance characteristics of the orbitrap can be achieved via increasing the electrostatic field, either by increasing the applied voltages or by altering the orbitrap geometry. As these performance characteristics can be achieved with a purely electrostatic field, the orbitrap eliminates the need for a superconducting magnet, and consequently, the disadvantages that arise from a superconducting magnet.

**Compound identification: accurate mass**

Once a mass spectrum is generated, compound identification is conducted for unknown ions with relevant distributions within the sample. Although MALDI MS often provides the molecular weight of biomolecules, the nominal molecular weight alone is not suitable for compound identification; a number of different compounds can produce ions at the same nominal \( m/z \). For further identification, there are two mass spectrometric strategies: 1) obtaining accurate mass measurements and 2) performing tandem mass spectrometry (MS\(^n\)). Accurate mass measurements, assuming the data are collected on a well-calibrated instrument, provide information about the collective mass defect of the ion. Based on the mass defect, an empirical formula for the ion in question can be calculated. This strategy is often employed on high-resolution ToF mass analyzers and Fourier transform mass analyzers. Accurate mass measurements,
however, cannot provide structural information to differentiate isomeric compounds (i.e., compounds that have same empirical formula, but differ in structural configuration).

**Compound identification: tandem mass spectrometry**

Tandem mass spectrometry ($\text{MS}^n$) provides complementary information to accurate mass studies. In this strategy, an ion of interest is isolated, and energy is imparted upon the ion. Eventually, the internal energy of the ion becomes great enough that labile bonds begin to break, resulting in lower mass fragment ions compared to the initial precursor ion. Based on the fragmentation pattern, one can deduce the initial structure of the precursor ion.

$\text{MS}^n$ can be conducted through a number of dissociation methods, including collision-induced dissociation (CID), electron-transfer dissociation (ETD), electron-capture dissociation (ECD), photodissociation (PD), and in-source fragmentation. Of these dissociation methods, CID has become the method of choice for dissociation of small molecules. In this method, energy is imparted by accelerating the precursor ion, resulting in energetic collisions with an inert collision gas. The collisions result in the conversion of a fraction of the precursor ion’s kinetic energy into internal energy. Provided enough kinetic energy is converted to internal energy, the precursor ion will fragment. Once a labile bond is broken, one side of the original precursor ion will often retain the charge, generating the fragment ion. The remaining side is commonly referred to as a neutral loss (NL), and is not directly detected.

**High-energy vs low-energy CID**

Although the fragment ions detected are largely compound dependent, the kinetic energy of the precursor ion also determines the generated fragments. High-energy CID is defined as a precursor ion having a kinetic energy of 1 keV or greater.\(^6\) In this
instance, the high-energy collisions with the relatively immobile collision gas result in a rapid increase in the internal energy of the ion. Low-energy CID is defined as a precursor ion that has a kinetic energy between 1 eV and a few hundred eV.\(^6\) In general, low-energy CID is utilized with triple quadrupole and ion trap mass analyzers, whereas high-energy CID has traditionally been utilized on tandem sector instruments.\(^6\)

**Tandem in space vs tandem in time MS\(^2\)**

MS\(^2\) can be performed either tandem in space or tandem in time, depending on the mass analyzer. Tandem in space MS\(^2\) is typically performed on either a triple quadrupole mass analyzer (QQQ) or other linear mass analyzer arrangement, such as a quadrupole time-of-flight mass analyzer (QToF) or tandem time-of-flight mass analyzer (ToF-ToF). In a QQQ, a precursor ion is mass selected in the first quadrupole, fragmented in the second quadrupole utilizing collisions with a neutral collision gas, and the fragment ions are mass analyzed in the third quadrupole. Tandem in time MS\(^2\) is typically performed within ion traps or other mass analyzers where the ions are stored within the analyzer region. In this scenario, selection of the precursor ion, fragmentation of the precursor ion, mass analysis of the resulting product ions all occur within the same trapping region, with the added benefit that fragmentation can be extended beyond the initial precursor ion. Thus, higher stages of mass spectrometry (i.e., MS\(^3\), MS\(^4\), etc...) can be performed without the need for additional mass analyzers.

**MS\(^n\) on the Thermo LTQ XL**

This section will discuss the theory and practical aspects of performing MS\(^n\) on a commercial linear ion trap mass analyzer, the Thermo LTQ XL (instrument schematic depicted in Figure 1-4). In general, MS\(^n\) on the linear ion trap consists of precursor ion isolation, collisional cooling of the precursor ion, ion activation (resulting in
fragmentation), and finally mass analysis and detection of the resulting fragment ions. Each of these events is detailed in Figure 1-5, and will be discussed in terms of the voltages and waveforms applied.

Ion isolation in a commercial Thermo LTQ XL is a two-step process. The first step in isolation is to shift the ion to be isolated to a q of 0.83. Recall that the LMCO of an ion trap is directly proportional to the RF voltage applied; thus, as a consequence of the first step of isolation, a number of low mass ions fall at a q with an unstable trajectory, that is at a q value past the LMCO at q=0.908. To eject the remaining ions, excluding the precursor ion, a sum of sines ion isolation waveform is applied that contains the resonant frequencies of all ions within the trap, except the precursor ion of interest. Following isolation of the precursor ion, a period of “cooling” is applied to the precursor ion population. In doing so, the RF voltage is decreased until the precursor ion is held at q=0.25. Furthermore, minor collisions with the helium damping gas remove kinetic energy from the ions, moving the ion population toward the center of the ion trap and ensuring that the precursor ion population has a stable trajectory within the trap.

Activation (fragmentation) of the ion population is then induced by applying a supplementary resonant excitation voltage across the exit rods. The overall magnitude of this excitation voltage is both compound and mass dependent; however, the Thermo LTQ XL uses a normalized collision energy (NCE%), which is a linear function that increases with increasing m/z. When applied, this voltage increases the precursor ion population’s motion in the radial direction, resulting in an increase in kinetic energy. As the kinetic energy of the population increases, energetic collisions will occur between the precursor ion and the helium damping gas. With enough time for energetic
collisions, the precursor ions will gain enough internal energy to fragment, typically along the lowest energy pathway. The resulting lower mass fragment ions will not undergo further fragmentation, as the excitation voltage is in resonance with the precursor ion population. It should be noted that broad-band excitation can be performed on the Thermo LTQ XL, which would yield further fragmentation of the fragment ions. Isolation and fragmentation can theoretically be repeated indefinitely assuming the fragment ions have stable trajectories within the trap and are amenable to fragmentation by CID.

Once all of the desired stages of MS\textsuperscript{n} have been performed, the fragment ions are ejected from the trap in order of increasing \( m/z \) for detection. To eject the ions, a combination of the main RF voltage and the supplementary resonant ejection voltage is utilized. Although the ions could theoretically be ejected with solely the main RF voltage, the supplementary resonant ejection voltage has been shown to improve the mass resolution in both the LIT and QIT.\textsuperscript{28,41} On the LTQ XL, the resonant ejection waveform is in resonance when the ions to be ejected fall at \( q=0.88 \).

**Data processing**

**Image generation**

Mass spectrometric images are generated by extracting the intensity of a specified MS or MS\textsuperscript{n} \( m/z \) range, and subsequently plotting that intensity vs. the X,Y position on tissue. There are a number of free software packages available for image generation. The most notable of these software packages is BioMap, a package originally developed for magnetic resonance imaging in 1996 and later adapted for MSI by Markus Stoeckli at Novartis.\textsuperscript{42} BioMap offers image generation with a number of statistical features, and supports a number of imaging dataset formats, including .msi
and .imzML. These two file formats are sufficient for most instrument platforms (except Thermo Scientific imaging instruments), as there are free converters for both .msi and .imzML available. At present, a converter exists for converting Thermo Scientific .raw files to .imzML files; however, the converter is functional only for datasets that conform to specific constraints (i.e., images collected with rectangular dimensions). Thermo Scientific developed Thermo ImageQuest to support imaging experiments conducted on all Thermo instruments. This software uses two file formats in concurrence, the .raw file containing the mass spectra and a .MALDImpos file that associates the relative position with each mass spectrum. Although sufficient for basic image generation, ImageQuest lacks functionality for more complex statistical analysis.

**Normalization**

The MALDI ionization process is thought to be the largest source of signal variability in a MALDI MSI experiment. Unfortunately, a number of sources of variability exist in this process. For instance, heterogeneous application and crystallization of the MALDI matrix will contribute to fluctuation in ionization yields. Also, the local sample topography can also introduce variability, as relatively small changes in height can cause variations in laser fluence delivered to the sample. Finally, the shot-to-shot variability in the laser can also contribute to fluctuation in ionization yields.

To account for this variability, normalization is often utilized for each individual pixel. To date, a number of strategies have been proposed, although normalization still remains a hotly debated topic in the MSI community. MSI normalization involves dividing the ion intensity of interest by either the total ion current (TIC), an internal standard, a matrix ion, or a target-specific ion that reflects the relative intensity at that pixel. An internal standard appears to be the optimal method for normalization;\textsuperscript{43-45}
however, endogenous signal at the same nominal mass of the internal standard in single-stage MS experiments may confound analysis. Furthermore, isotopically-labeled internal standards can be costly, and an internal standard may not demonstrate the same ionization efficiency as all analytes of interest in the target sample. Similarly, normalization to a matrix ion appears impractical, as the ionization efficiency of the matrix ion may not reflect the ionization efficiency of the analytes of interest.\(^{46}\) Finally, normalization to a target-specific ion assumes that there are no isobaric ions, and that the ion’s concentration does not vary throughout tissue. Currently, the simplest effective method for normalization appears to be dividing by the TIC. This method, however, is not without drawbacks, as the relative abundance of matrix and analyte ions may vary from pixel to pixel. Recently, researchers have proposed a novel normalization method that utilizes either the sum or median of ‘informative’ ions within the dataset.\(^{46}\) This method eliminates many of the aforementioned problems; however, rigorous statistical analysis is required to determine the group of ‘informative’ ions prior to normalization.

**Multivariate Data Analysis**

Over the past 10 years, there have been an increasing number of instruments capable of performing high mass resolution MSI. This increase in mass resolution has allowed researchers to analyze increasingly larger numbers of biological compounds by MSI than ever before. In particular, Fourier transform mass analyzers (e.g., orbitrap mass analyzers or Fourier transform ion cyclotron resonance cells), in which resolving power is theoretically proportional to the transient time,\(^{39}\) present a promising opportunity for discriminating and imaging nominal isobaric compounds. Unfortunately,
an increase in mass resolution is invariably linked to an increase in dataset size, and consequently, analysis time.

As a case study, let’s consider two isobaric phosphatidylcholine (PC) ions commonly detected in positive-mode MALDI MS studies of mammalian tissue: the [M+H]⁺ of PC (16:0/20:4) and the [M+Na]⁺ of PC (16:0/18:1). Nominally, both ions appear at m/z 782; however, the two ions have exact masses of m/z 782.5694 and 782.5676 for PC (16:0/20:4) and PC (16:0/18:1), respectively. To resolve these two lipid ions, the mass analyzer would need a resolution of approximately 500,000, and would similarly need to collect data at 0.001 amu intervals. If data of this nature were collected in the mass range of 150–2000 amu, a common mass range used for lipid and metabolite MALDI mass spectrometry, a total of 1,850,000 possible images could be generated. Assuming a researcher could effectively analyze a single image each second, it would take the researcher approximately three months to thoroughly analyze the entire dataset. The time-consuming nature of fully interpreting a high-resolution MSI dataset stems from its inherent multidimensionality; the number of dimensions in the dataset is equal to the number of variables or mass features, in this test case, 1,850,000. Thus, a more efficient method of interpreting highly-dimensional datasets would be beneficial to the MSI community.

**Principal component analysis**

Multivariate data analysis techniques, such as principal component analysis (PCA) are methods utilized to reduce the dimensionality of datasets. These data reduction techniques serve to calculate the axis of greatest variation, also known as the first component, through the multidimensional space of a dataset. The second component is then orthogonal to the first, and the process can be repeated for as many principal
components as there are variables. The resulting components consist of linear combinations of weighted variables. Variables that contribute greatest to the variance within a dataset are given relatively large weighting factors (loadings), and variables that have minor contributions are given relatively small weighting factors. A sample’s score is represented by the summation of the products of a variable and its corresponding loading coefficient. The samples in the dataset can then be projected onto component axes by plotting the scores in 2 or 3 dimensions (also known as a scores plot). Theoretically, the scores plot can be extended to as many components as there are variables; however, 2 or 3 dimensions are all that can be effectively visualized by humans. Furthermore, if the dataset contains a sufficiently high number of variables, and significant correlations exist within the dataset, the first few principal components will account for a large percentage of the variation.\textsuperscript{47}

Although the concept of PCA appears simple, the data manipulation required and the mathematical principles that underlie PCA are more complicated. Prior to performing PCA, the sample data must first be transformed so that all variables are centered about the origin, commonly known as mean-centering. Next, although not required, it is recommended that the data are normalized so that unit variance is obtained. Once the data are transformed into a suitable format, one can begin to perform PCA. The first step in this process is to calculate the covariance matrix ($\Sigma$) of the variables, or alternatively the correlation matrix, depending on the normalization of the variables. Once $\Sigma$ is calculated, the mathematical formula of the $k^{th}$ principal component is simply given by Equation 1-15.

$$ z_k = \alpha'_k x $$

(1-15)
In this equation, \( \alpha'_k \) is simply an eigenvector of \( \Sigma \) corresponding to the \( k^{th} \) largest eigenvalue of the variance of \( z_k \), and \( x \) is a vector of the standardized variables.\(^{47}\) To maximize the variance of \( z_1 \), \( \alpha'_1 \Sigma \alpha_1 \) should be maximized; however, it should be noted that this maximum will only be found if a normalization constraint is applied, the most common being that the sums of squares of the elements within \( \alpha_k \) are equal to 1.\(^{47}\) This process is then repeated for \( z_2 \) using the same constraints, except that \( z_2 \) must be uncorrelated with \( z_1 \). The process is then extended for as many principal components as there are variables in the dataset.

A test data set for this type of analysis is displayed in Figure 1-6A. The data set contains ten school students in two sample groupings: 1\(^{st} \) grade students and 12\(^{th} \) grade students, and hypothetical data for the students’ height (cm) and weight (kg) is shown. Initially, the averages for all variables are calculated, and individual data points are mean-centered (Figure 1-6B). Following mean-centering, a 2-dimensional plot can be generated to visualize the sample groupings in Euclidean space (Figure 1-6C). From this plot, it is clear to see that the direction of greatest variance within the dataset is a line passing through the center of both sample groupings. This direction, depicted in the Figure 1-6C as a green line, is the first principal component in PCA, and demonstrates variability between the sample groupings. The second principal component, by definition, is uncorrelated (read orthogonal in a graphical sense) to the first principal component, and is represented by a red line in Figure 1-6C. This principal component largely demonstrates variability within a sample grouping. By rotating the 2-dimensional space so that the principal components now fall on the x- and y-axes, the data are projected into a 2-dimensional scores plot (Figure 1-6D). The loadings,
represented by $\alpha_k$ for the mathematical example above, that contribute to this separation can then be examined to determine the features that drive the separation in the data set. Although this is a trivial exercise with just 2 or 3 variables, extending this exercise to further variables yields complex calculations and a data space that humans are incapable of visualizing. Fortunately, modern computers can perform complex PCA calculations and rotations within a multidimensional data set containing thousands of variables within seconds, allowing for separation of sample groupings to be visualized in 2- or 3-dimensional principal component axes. Furthermore, a number of open-source algorithms have been written to make these calculations readily available to the scientific community.

**Partial least squares discriminant analysis**

Similar to PCA, partial least squares discriminant analysis (PLS-DA) also determines the direction of maximum variance within a dataset. The major difference between PCA and PLS-DA is that PLS-DA determines the axis of greatest variation based on the covariance as determined by the data and sample groupings.\(^{48, 49}\) This type of multivariate analysis is considered a supervised analysis, and is often used to develop a classification model. Furthermore, PLS-DA serves to eliminate variance that is unrelated to sample groupings (e.g., sample preparation or unrelated biological variability).

**Applying multivariate data analysis to MSI**

Although PCA has primarily been utilized in chromatographic applications of mass spectrometry, there has been recent interest to utilize PCA with MSI applications.\(^{48, 50}\) In general, there are two different approaches for adapting MSI datasets for use with PCA. The simpler approach is a guided methodology, wherein individual regions of
interest (ROIs) within the dataset are averaged for PCA. This approach allows the researcher a measure of control over the dataset, as unwanted pixels or regions of pixels can be omitted. Although this approach may simplify PCA interpretation, there is the potential for researchers to overlook valuable information.

In contrast, the second approach is an unbiased method, wherein each pixel, or alternatively a specified number of averaged pixels, is treated as an individual sample. In this method, the only excluded pixels are those corresponding to areas off of the target sample (background pixels). The second approach has the advantage of the ability to plot scores images, wherein the score of a particular principal component can be plotted in lieu of intensity for each pixel. Furthermore, ion distributions that may have been overlooked using the guided approach may be found with this unbiased approach. The main disadvantage of the unbiased approach is that the inclusion of pixels not corresponding to ROIs may confound PCA analysis and interpretation. If possible, a combination of both the above approaches is recommended when interrogating an MSI dataset. At present, the main hindrance for this depth of analysis is the lack of suitable methodologies and available software, both of which will be addressed during this work.

**Scope of the Dissertation**

The remainder of this dissertation provides methodologies for integrating MSI and multivariate data analysis as applied to two biological applications. Chapter 2 will detail the use of PCA and PLS-DA as applied to positive-mode MALDI MSI datasets collected from a model system for myocardial infarction. In this work, the ability of both PCA and PLS-DA to efficiently determine region-specific markers will be highlighted. Furthermore, the relative merits of two PCA methodologies (guided and unbiased) will be explained. Chapter 3 will discuss the use of 9-aminoacridine as a matrix for both
positive- and negative-mode imaging of the same biological system as introduced in Chapter 2. This chapter will highlight the ability of a basic matrix to expand the breadth of detectable analytes by MALDI MS. Furthermore, the methodologies developed in Chapter 2 will be used to determine novel tissue-specific markers for myocardial infarction. The final research chapter, Chapter 4, explores the utility of MALDI MSI to analyze whole organisms without any prior sample preparation other than matrix coating. The methodologies developed in Chapter 2 will then be utilized to validate this approach through the analysis of genetic mutants. Finally, Chapter 5 provides a brief summary of the research performed, the future work that will be conducted, and the outlook for MALDI MSI as coupled to multivariate data analysis.
Figure 1-1. Representation of the MALDI process (Adapted from Chughtai et al.)\textsuperscript{2}. Dimensions are not drawn to scale.
Figure 1-2. Positive portion of the first stability region for a quadrupole mass filter. Regions of stability within the quadrupole mass filter are shaded in grey. A theoretical operating line for a quadrupole mass filter is also shown (red) with ions of varying $m/z$ in green. (Adapted from Douglas et al.)\textsuperscript{51}
Figure 1-3. Sample stability diagram expressed in terms of $a_z$ and $q_z$ for a QIT. Regions of stability are shaded in grey. Furthermore, the LMCO for a conventional QIT operated in RF-only mode is designated. (Adapted from March)\textsuperscript{52}
Figure 1-4. Schematic of the Thermo Scientific LTQ XL with a MALDI ionization source. (Adapted from Garrett et al.)\textsuperscript{31}
Figure 1-5. Thermo Scientific LTQ XL tandem MS\textsuperscript{n} scan function. The three applied waveforms (main RF, resonant excitation, and ion isolation) for precursor ion isolation (red), precursor ion collisional cooling (blue), precursor ion activation (orange), and fragment ion ejection (green) are displayed.
Figure 1-6. Example of PCA applied to 2-variable dataset. The data are expressed in terms of A) raw values and B) mean-centered values. Additionally, the data are plotted in relation to C) the two variables and D) the first two principal components generated by PCA.
CHAPTER 2
MALDI MASS SPECTROMETRIC IMAGING OF CARDIAC TISSUE FOLLOWING CORONARY ARTERY LIGATION INDUCED MYOCARDIAL INFARCTION

Introduction

Coronary heart disease (CHD) has remained the number one cause of death in the United States over the past four decades. Many deaths occurring in patients with CHD arise from acute myocardial infarction (MI), more commonly known as a heart attack. The onset of MI is most often the result of atheromatous occlusion of coronary arteries restricting blood supply to cardiac tissue. The lack of oxygenated blood to cardiac tissue results in severe and often irreversible damage that may ultimately lead to heart failure. Although MI affects millions of people each year, the blood-borne and tissue-specific biochemical changes that occur following MI are not fully characterized, and few of the known biochemical markers are utilized in a clinical setting. To date, increases in two sets of blood-borne protein markers, creatine kinase (CK-MB) and troponins, are commonly used for the detection of myocardial necrosis (tissue death) following MI; however, these biomarker concentrations return to normal levels within days. The discovery of a robust biomarker would improve both clinical diagnosis capabilities and our understanding of this condition. Furthermore, identifying biochemical changes in infarcted myocardium may provide mechanistic insights for new therapeutic intervention targets designed to limit myocardial tissue loss. Thus, there is a significant need to study the biochemical changes resulting from both CHD and MI.

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Mass spectrometry (MS) is an analytical tool with the potential to provide mechanistic revelation for CHD characterization, biomarker discovery, and clinical diagnosis. MS was utilized as early as 1996 to characterize protein epitopes for MI as well as alterations in fatty acid metabolism in ischemic myocardium. Since these early studies, the focus of CHD MS research has shifted to proteomic and metabolomic analyses of plasma, serum, and urine revealing a number of proteins and metabolites as potential biomarkers and/or predictors for MI resulting from CHD. In addition, an extensive list of potential protein markers in serum was recently compiled, with the most abundant 25% projected to be amenable to tandem MS platforms. Although there has been extensive study on biological fluids, MS analysis of intact cardiac tissue from CHD and MI positive specimens has not been thoroughly explored. Thus, an analytical method that characterizes small molecule biochemical changes in intact cardiac tissue following MI may yield valuable insight. Furthermore, if this method can provide localization of these relevant biomolecules, a deeper understanding of the biochemistry underlying MI may be obtained.

Mass spectrometric imaging (MSI) is a microprobe technique that generates chemically selective images from thin tissue sections. The most common ionization technique for MSI, matrix-assisted laser desorption/ionization (MALDI), is a soft ionization technique well-suited for the analysis of small and large biomolecules. The coupling of MALDI and MSI allows for tissue imaging by rastering a sample with respect to a stationary laser beam, collecting mass spectra at discrete positions. An image is then generated by plotting the intensity of a selected mass-to-charge (m/z) versus the X,Y position, thereby providing both chemical and spatial information. To date, MALDI
MSI analyses of intact tissue have identified the spatial distribution of endogenous and exogenous compounds including proteins,\textsuperscript{1, 67} peptides,\textsuperscript{22, 68} lipids,\textsuperscript{31, 69, 70} and drugs.\textsuperscript{71-73} One advantage of this technique is the ability to elucidate relative intensity changes and spatial distributions resulting from external stimuli such as administration of an exogenous drug\textsuperscript{71} or injury models.\textsuperscript{74} Thus, MALDI MSI should be an ideal tool to delineate local alterations in lipids and metabolites in infarcted myocardium following a model such as a left anterior descending (LAD) coronary artery ligation. This model permits comparison of perfused (non-affected) and affected zones of tissue within a single section, eliminating much of the variability inherent in tissue-to-tissue comparisons.

Although MALDI MSI tissue experiments can generate a wealth of data, interpretation of multi-dimensional data sets can be time-consuming, tedious, and/or inconclusive. Multivariate data analysis has long been a staple of the ‘omics’ workflows, and has recently become of interest in the MSI community.\textsuperscript{75-77} Common multivariate techniques such as principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) can be utilized for data reduction in multi-dimensional data sets. Briefly, these techniques group samples, or regions of tissue for MSI, based on linear combinations of weighted variables, known as components. Although PCA and PLS-DA both determine the direction of maximum variance within a data set, the techniques’ definitions of maximum variance differs. PCA is an unsupervised technique that finds directions maximizing the total variance within a data set, whereas PLS-DA is a supervised technique that separates samples based on the covariance determined by both the data set and membership groupings.\textsuperscript{49} For visualization, samples are
represented on two-dimensional or three-dimensional scores plots, and the weighting factors for each variable are displayed on loadings plots. Variables with the largest weighting factors contribute significantly to the grouping separation; thus, data sets containing large numbers of variables are reduced to a specified number of variables, simplifying data interpretation.

This work reports the combined use of MALDI MSI and two multivariate data analysis methodologies to study lipids and metabolites in cardiac tissue following LAD coronary artery ligation. Initially, a guided multivariate data analysis methodology that interrogates user-selected regions of interest from the MSI dataset is introduced to identify tissue-specific markers of these regions following MI. In addition, an unbiased multivariate data analysis methodology is introduced for more exploratory analysis of MSI datasets, and the relative merits of each methodology are discussed.

Experimental

**Chemicals and reagents**

2,5-dihydroxybenzoic acid (DHB) and phospholipase A\(_2\) (PLA\(_2\)) from bovine pancreas were purchased from Sigma-Aldrich (St. Louis, MO). Sodium acetate (NaOAc), anhydrous creatine, HPLC-grade water (H\(_2\)O), and HPLC-grade methanol (MeOH) were purchased from Fisher Scientific (Fair Lawn, NJ). 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC 17:0) was purchased from Avanti Polar Lipids (Alabaster, AL). 100% ethyl alcohol (EtOH) was purchased from Decon Labs (King of Prussia, PA). A creatine standard was prepared in H\(_2\)O to a final concentration of 100 ppm. Additionally, the LPC 17:0 standard was dissolved and diluted in EtOH to a final concentration of 100 ppm. PLA\(_2\) was dissolved in H\(_2\)O and diluted to a final concentration of 500 ppm. A MALDI matrix solution consisting of 40 mg/mL DHB in
70:30 MeOH:H₂O (v/v) was prepared for the analysis of creatine (matrix 1). A second matrix solution was prepared as above, except sodium acetate (NaOAc) was added to a final concentration of 10 mM for the analysis of lipids (matrix 2).

**Biological sample preparation**

All animal procedures were conducted in accordance with guidelines published in the *Guide for the Care and Use of Laboratory Animals* (National Research Council, National Academy Press, Washington, DC, 2010) and were approved by the Animal Care Committee of Saint Louis University. Ligation of the LAD was performed as previously described. In brief, male Sprague-Dawley rats (250–300 g body weight) were injected with ketamine/xylazine (55 mg/mL, 7 mg/mL; 0.1 mL/100 g, i.p.). Rats were subsequently intubated and injected with Buprenex (0.05 mg/mL; 0.1 mL/100 g, i.p.). Animals were then ventilated with air at a tidal volume of 3–4 mL and a rate of 50–60 breaths/min (Harvard Apparatus). A left lateral thoracotomy was then performed. The thoracic cage was exposed and the intercostal space between ribs 4 and 5 was separated with a retractor. The left atrial appendage was retracted and a 6-0 suture was placed around the proximal LAD. This LAD suture was tied tightly or loosely to produce an infarction (6 animals) or sham surgery (6 animals), respectively, and then the thoracic incision was closed with a 5-0 suture. After recovery from surgery, rats were weighed and individually housed. Twenty-four hours following LAD occlusion, rats were euthanized with pentobarbital (~800 mg/kg, i.p.) and subsequently hearts were removed and flash-frozen in liquid nitrogen. All organs were stored at -80 °C until further preparation.

Heart tissue was bisected along a transverse plane that passed through the left and right ventricles. The upper half of the heart was subjected to 2,3,5-
triphenyltetrazolium chloride (TTC) staining to distinguish perfused and damaged tissue. The remaining lower half of the heart was utilized for MALDI MSI analysis. Cardiac organs were sectioned using a Microm HM 505E cryostat (Waldorf, Germany). Optimal cutting temperature polymer (OCT) was not used for tissue mounting, as OCT is reported to produce abundant ion signals in MALDI analysis of thin tissue sections, resulting in analyte ion suppression. Instead, organs were held atop a drop of HPLC-grade H₂O on the cutting stage and placed into the cryostat chamber held at -25 °C, thereby freezing the drop of water and fusing the organ to the cutting stage. Subsequent 10-µm thick sections were thaw-mounted atop glass microscope slides and stored at -80 °C. Prior to matrix coating, tissue sections were placed in a vacuum desiccator for approximately 45 minutes to remove excess H₂O.

Serial cardiac sections were coated with either matrix 1 or matrix 2. MALDI matrix was spray coated atop tissue sections using a glass Type A Meinhard nebulizer (Golden, CO). Nitrogen was used as the nebulizing gas at a pressure of 30 PSI and the matrix solution was delivered at a flow rate of 3 mL/min. In this method, approximately six passes were conducted over each slide followed by 15 seconds of waiting time in order to avoid excessive wetting of the tissue sections. The process was repeated until a homogenous layer of matrix crystals was obtained over the entire tissue. Approximately 8 mL of MALDI matrix solution (320 mg of DHB) were used per microscope slide to obtain a suitable matrix layer on the tissue sections.

**Instrumentation**

Experiments were performed using either a Thermo Scientific LTQ XL linear ion trap mass spectrometer (San Jose, CA) or a Waters Synapt (Milford, MA). All experiments were performed on the LTQ XL unless otherwise noted. The LTQ XL was
equipped with a MALDI ionization source, consisting of a Lasertechnik Berlin MNL 106-LD N\textsubscript{2} laser (\(\lambda=337\text{nm}\)) (Berlin, Germany). This laser has a repetition rate of 60 Hz and produces a laser spot diameter of approximately 100 \(\mu\text{m}\). The Waters Synapt was equipped with a MALDI ionization source consisting of a frequency tripled Nd:YAG laser (\(\lambda=355\text{nm}\)). This laser has a repetition rate of 200 Hz and produces a laser spot diameter of approximately 75 \(\mu\text{m}\).

**Standard analysis**

Standards were deposited atop a 96-well stainless steel MALDI plate using a modified dried-droplet method.\textsuperscript{3} In this method, 1 \(\mu\text{L}\) of the prepared standard followed by 1 \(\mu\text{L}\) of Matrix 1 (for creatine) or Matrix 2 (for lipids) were pipetted onto the well plate. Solvent was evaporated from the mixture using gentle heat, leaving behind MALDI matrix and analyte co-crystals. MS and MS\textsuperscript{2} spectra were acquired using a laser energy of 5 \(\mu\text{J}\) and 3 laser shots per laser stop, and 100 spectra were averaged to produce one standard MS or MS\textsuperscript{2} spectrum.

**Tissue analysis**

Following analysis of standards, MSI was performed on prepared cardiac tissue sections. The tissue sections were rastered with respect to the laser at a horizontal and vertical step size of 100 \(\mu\text{m}\). MS spectra (\(m/z\) 100–250) were collected in profile mode over tissue sections coated with Matrix 1. Additionally, MS spectra (\(m/z\) 200–2000) were collected in centroid mode (to minimize the size of the data files) over tissue coated with Matrix 2. A laser energy of 4 \(\mu\text{J}\) and 3 laser shots per laser stop were utilized for all MS and MS\textsuperscript{2} imaging experiments. Images were generated using Thermo ImageQuest v1.0.1 software. All MS images were normalized to the total ion current (TIC), but normalization was not conducted for MS\textsuperscript{2} images.
Compound identification for lipid species from cardiac tissue was performed using tandem MS with collision-induced dissociation (CID) or pulsed Q collision-induced dissociation (PQD). For MS² experiments, an isolation width of 1.2 amu and a collision energy of 35 AU (arbitrary units normalized to m/z 400) was utilized. In instances where the linear ion trap’s low-mass cutoff hindered ion identification, PQD was utilized with an isolation width of 1.2 amu and a collision energy of 25 AU.\(^1\) In instances where MS³ was necessary to identify ions, the same settings were utilized for the second stage of MS, and the third stage of MS was performed with an isolation width of 1.5 amu and a collision energy of 35 AU.

**Statistical analysis**

Multivariate data analysis and significance testing were performed to determine significant m/z values (features) differing between viable and infarcted tissue within the same tissue section. In doing so, two different methodologies were applied: 1) a guided multivariate data analysis methodology and 2) a supervised multivariate data analysis methodology.

For the guided methodology, PCA and PLS-DA were performed using Metaboanalyst web server.\(^{49,82}\) Five samples, each consisting of an average of 25 mass spectra, were selected from each tissue region. Lists of mass-to-charge (from centroid data) and intensity were exported from QualBrowser into Microsoft Excel\(^{©}\) and saved as .csv files. Since Metaboanalyst does not support mean-centering without further normalization (e.g., normalization to the range or standard deviation of the mean), the data were processed in two different methods: the first method mean-centered the intensity at each m/z value before it was imported into Metaboanalyst and the second method left the data unprocessed before it was imported. After the
processed \( m/z \) and intensity lists were imported, a mass tolerance of 0.75 \( m/z \) was utilized to counteract possible mass shifts due to space charge effects. Next, \( m/z \) values known to arise from MALDI matrix ions were removed from all samples. In this case, the intense matrix ions observed in the mass spectra were \( m/z \) 273 and 274, representing the \([2M-2H_2O+H]^+\) ion and its \(^{13}\text{C}\) isotope, respectively. Following MALDI matrix peak removal, signal intensities were normalized to the TIC within each sample to account for signal variability inherent in MALDI MS tissue analysis. Additionally, the utility of scaling techniques (e.g., autoscaling) was investigated. After multivariate data analysis, a Student’s t-test was performed on selected features using Microsoft Excel.

For the unbiased multivariate data analysis methodology, PCA was performed using the ChemomeTricks tool box developed at FOM Institute AMOLF. As opposed to averaging pixels from each region to define a ‘sample’, each pixel was treated as a separate sample. For files collected on the Thermo LTQ XL, the .RAW file was first converted to .NetCDF using the Roadmap File Converter provided with Xcalibur. The data in the .NetCDF file were extracted with in-house software, and saved as a .MAT file containing pertinent spatial and spectral information. Due to the large amount of data present in this dataset, peak selection and alignment were performed. For this process, a summed spectrum of all pixels was created. Next, baseline subtraction was performed to enhance the signal-to-noise ratio of relevant mass features. To further reduce the size of the dataset, a user-selected peak threshold (typically 0.1–15% of the base peak area) was applied, and all mass features falling below this threshold were excluded. The remaining data were then saved as a .mat file. Following peak picking and alignment, a first iteration of PCA was performed. Pixels corresponding to areas off
tissue, and ions with high loadings coefficients in these pixels (matrix ions), were then removed. A second iteration of PCA was then performed, and scores images were generated for the first 20 principal components. Loadings plots were then generated for all principal components that demonstrated relevant localization. Finally, clustering analysis using a user-selected number of principal components was performed.

**Enzymatic digestion with PLA$_2$ on tissue**

The in vitro action of PLA$_2$ was also explored on sham surgery cardiac tissue. In this method, 5 $\mu$L of 500 ppm PLA$_2$ in water was spotted on freshly sectioned tissue (before desiccation). The volume and concentration of PLA$_2$ was chosen so that approximately half of the tissue contained completely digested phospholipids. The tissue was then incubated at room temperature for one hour. Afterwards, the tissue was placed in a vacuum desiccator for one hour. As a control, the same procedure was conducted with the exception that HPLC-grade H$_2$O was spotted atop tissue in lieu of PLA$_2$. Following desiccation, the tissue was coated with matrix 2 as described above. Coated tissue was then introduced into the mass spectrometer. Representative mass spectra, consisting of an average of 100 scans, were collected from both tissue samples. MS imaging experiments were also conducted over both tissues.

**Results**

**Identification of infarcted myocardium**

**TTC staining**

Prior to preparation for MSI analysis, the upper half of selected cardiac organs was submitted to TTC staining. This staining protocol is traditionally utilized to identify regions of tissue damage. In its original state, TTC is colorless; however, in the presence of dehydrogenases from mitochondria of healthy tissue, enzymatic reduction
alters TTC to a formazan, producing a brick red color (Figure 2-1A). Infarcted tissues lack the functioning mitochondria necessary for enzymatic reduction; thus, the tissue remains unstained. TTC staining of the heart (Figure 2-1B) illustrated infarcted myocardium in the affected area of the left ventricle, which was previously supplied arterial blood by the LAD.

**Creatine**

Biochemically, creatine kinase (CK) consumes one molecule of adenosine triphosphate (ATP) to catalyze the conversion of one molecule of creatine to one molecule of phosphocreatine, which serves as a high energy phosphate buffer for ATP in muscle tissue. Three to six hours following a heart attack, CK-MB, a creatine kinase selective to cardiac tissue, is released into the interstitial fluid, and consequently the bloodstream, resulting in an elevated plasma level of CK-MB. Although CK-MB can be detected using biological assays, the molecular weight of CK-MB (~86 kDa) and the limited mass range of a linear ion trap hinder direct detection of this enzyme. Conversely, the enzyme’s substrate, creatine, is well within the mass range of the linear ion trap. One might expect decreased levels of creatine within infarcted or damaged tissues if the damaged tissue also leaks water-soluble metabolites such as the substrate of CK.

Prior to tissue analysis, a creatine standard was characterized in MS and MS$^2$ modes. MS analysis yields analyte ions at $m/z$ 132 and $m/z$ 154 corresponding to the [M+H]$^+$ and [M+Na]$^+$ ions, respectively. The ion at $m/z$ 154 is isobaric with the [M]$^+$ for DHB; thus, the addition of sodium acetate to the MALDI matrix would not be beneficial for MS studies of creatine, as it may drive creatine ion signal to [M+Na]$^+$. The MS$^2$ spectrum of $m/z$ 132 demonstrates a single abundant fragment ion at $m/z$ 90.
(protonated n-methylglycine, more commonly known as sarcosine) resulting from the loss of CH₂N₂.

Following authentic creatine characterization, MS and MS² were applied to assess the spatial localization of creatine in cross-sectional tissue sections from hearts sliced at the mid-ventricle level. Both control rats and rats subjected to 24h of regional ischemia as a result of LAD coronary artery occlusion were analyzed. The MS image of m/z 132 normalized to the TIC (Figure 2-1C) demonstrates a lower signal in the infarcted region of tissue, positively correlating with the TTC image (Figure 2-1B). Similarly, the MS² image mapping the transition from m/z 132 → 90 also demonstrates a lower signal in the infarcted region of tissue (Figure 2-1D). In contrast, control tissues demonstrated a constant signal throughout cardiac tissue sections (Figure 2-2). The lower creatine signal may reflect the state of the plasma membrane of cells within infarcted myocardium. Upon rupture of these plasma membranes, water-soluble enzymes (e.g., CK) and possibly small metabolite substrates such as creatine may leach out from infarcted myocardium. Similar to what is observed with CKMB following MI, a concurrent increase in blood-borne creatine concentration would be expected.

**Phospholipase A₂**

Phospholipase A₂ (PLA₂) is an enzyme that hydrolyzes the sn-2 acyl bond of intact phospholipids (PLs) including phosphatidylcholines (PCs) and phosphatidylethanolamines (PEs), yielding free fatty acids and lysophospholipids (lysoPLs) including lysophosphatidylcholines (LPCs) and lysophosphatidylethanolamines (LPEs). Multiple investigations have suggested an important role of increased PLA₂ activity as an enzymic mediator of the
pathophysiological sequelae of myocardial ischemia.\textsuperscript{85-87} Although LPCs and LPEs have been considered important arrhythmogenic lipids generated by PLA\textsubscript{2},\textsuperscript{88} the spatial localization of these lysoPLs in ischemic myocardium has not been investigated. Accordingly, MSI experiments were performed on tissue sections of hearts subjected to LAD coronary artery ligation. Representative mass spectra (an average of 50 scans) from the lipid region ($m/z$ 450–900) for infarcted and perfused tissue are shown in Figure 2-3. An increase in relative intensity is observed for various ions in the lysoPL region ($m/z$ 450–600) of the infarcted zones of these sections; however, few other major differences were elucidated from the averaged mass spectra from the two zones.

To confirm that the above ions in the lysoPL region resulted from the enzymatic action of PLA\textsubscript{2}, in vitro digestion of control myocardium (sham surgery) was performed. In doing so, freshly sectioned myocardium was exposed to approximately 5 \textmu L of 500 ppm PLA\textsubscript{2} for 1 hour prior to desiccation. The tissue section was then desiccated, prepared, and analyzed as above. Representative mass spectra from the lipid region of the mass spectrum are displayed for both the digested and control myocardium in Figure 2-4. A manual comparison between the lysoPL regions in Figures 2-3 and 2-4 reveals a striking resemblance between the infarcted myocardium from the ligation model (Figure 2-3A) and the digested myocardium from the sham model (Figure 2-4A), suggesting that these markers of infarcted (necrotic) tissue are formed via the action of PLA\textsubscript{2}. Similarly, the control myocardium in both models (perfused myocardium in the ligation model and myocardium without enzymatic modification in the sham model) do not demonstrate appreciable ion signal in the lysoPL region; however, abundant ion signal is observed in the intact PL region for both models.
Identification of At-Risk Myocardium: Triacylglycerides

Triacylglycerides (TAGs) have also been shown to be increased in response to MI, which likely reflects inefficient mitochondrial beta oxidation of fatty acids during ischemia.\(^9\) Oil red O-staining conducted on hearts following myocardial infarction has identified lipid droplets in the at-risk myocardium (i.e., the region surrounding infarcted tissue).\(^9\) It has been hypothesized that these lipid droplets are largely composed of TAGs and cholesteryl esters.\(^5\) In order to verify the hypothesis that these lipid droplets contain elevated concentrations of TAGs, MS\(^2\) imaging experiments were conducted over infarcted cardiac tissue.

It was recently reported that high concentrations of PCs hinder the detection of other classes of lipids by MALDI MS;\(^9\) thus, MS\(^2\) is often necessary for the detection of low abundance TAGs. MS\(^2\) analyses of alkali metal adducts (e.g., [M+Na]\(^+\)) of TAGs reveal abundant fragmentation relating to the three fatty acid tails. A representative MS\(^2\) spectrum of \(m/z\) 881 from cardiac tissue is shown in Figure 2-5A. Abundant fragment ions are observed at \(m/z\) 625, 601, 599, and 597, arising from NLs of 256, 280, 282, and 284 Da, respectively. These NLs indicate two isobaric TAG ions at \(m/z\) 881, the [M+Na]\(^+\) of TAG (16:0/18:1/18:1) and the [M+Na]\(^+\) of TAG (16:0/18:0/18:2). The two most abundant fragments, the loss of the 18:1 tail at \(m/z\) 599 (NL of 282) and the loss of the 16:0 tail at \(m/z\) 625 (NL of 256), are approximately twice as intense as the loss of the 18:0 and 18:2 tails at \(m/z\) 597 and 601, respectively, reflecting the relative abundance of the fatty acid tails within these isobaric TAGs.

MS\(^2\) imaging experiments over infarcted cardiac tissue sections were conducted on commonly observed TAGs, namely TAG 50:2, TAG 52:3, TAG 52:2, and TAG 54:3 present as sodiated species at \(m/z\) 853, 879, 881, and 907, respectively. Images were
generated for the expected neutral losses from each of the TAG species. The images for two of the fragment ions from m/z 881, the loss of the 16:0 tail at m/z 625 (NL of 256) and the loss of the 18:1 tail at m/z 599 (NL of 282), are shown in Figures 2-5B and 2-5C. Intense ion signal is observed surrounding the area of infarcted tissue, correlating well with the lipid droplets observed in electron microscopy. The localization of TAGs in the area surrounding infarcted tissue supports the hypothesis that lipid droplets following MI contain elevated levels of TAGs.

**Guided multivariate data analysis and identification of tissue-specific markers**

**Infarcted and perfused myocardium**

PCA and PLS-DA were conducted on both tissue zones of a single infarcted section. A single tissue section was chosen to minimize any variance that might occur from section to section or specimen to specimen. Multiple samples were generated from a single cardiac section by choosing five horizontal lines, each line consisting of 25 mass spectra, within a selected tissue region. Scores plots from PCA and PLS-DA, shown in Figure 2-6, were generated using Metaboanalyst. Separation between perfused and infarcted myocardium was observed in both multivariate data analysis techniques. In PCA of the mean-centered and TIC-normalized data, principal component 1 (PC 1) and principal component 2 (PC 2) carried 85.8% and 7.2% of the total variance, respectively (Figure 2-6A). The separation between infarcted and perfused tissue was largely dictated by PC 1, whereas PC 2 accounted for the variance within the sample groupings. The autoscaled PCA plots demonstrated a similar separation (data not shown).

In contrast to PCA, PLS-DA is a supervised technique that separates samples based on the largest covariance in the data set (i.e., the sample groupings are known).
The PLS-DA scores plot for the TIC-normalized peak list is shown in Figure 2-6B. Similar to PCA, component 1 carries 85.8% of the covariance, and sufficiently separates the two tissue zones. Although the PCA and PLS-DA scores plots show similar separation along the first component, PLS-DA demonstrated a tighter cluster within each zone along the second component. PLS-DA was also conducted on the autoscaled data (data not shown). The generated scores plot also shows sufficient separation between the perfused and infarcted zones along component 1.

The loadings plots for these four separation methods were then analyzed for significant m/z values that influenced separation (Figure 2-7). In all four methods, the separation was dictated by PC 1 (for PCA) or component 1 (for PLS-DA). Thus, analysis of the loadings plots can be simplified to just a single PC or component for identifying significant m/z values. When analyzing the loadings for the mean-centered, TIC-normalized data without autoscaling (Figure 2-7A), m/z values in the lysoPL region were positively correlated with infarcted tissue (e.g., m/z 496, 524, 546, and 562). In contrast, some m/z values present in the intact PL region were positively correlated with the unaffected perfused tissue (e.g., m/z 810 and 848). Analysis of the loadings for the autoscaled data proved to be more difficult, as many m/z values proved to have similar loadings values (Figure 2-7B). In principle, any variable that differs significantly between sample sets should influence the separation; however, a major drawback for autoscaling is that low intensity signals, which may have large relative variances, are weighted equally with high intensity variables. Upon analysis of the autoscaled loadings values, the majority of the m/z values with high loadings values appeared to be of low
intensity; therefore, autoscaling was not utilized for feature identification. Loadings plots from the PLS-DA data produced many of the same m/z values as PCA.

Significance testing was then performed on selected m/z values identified by PCA and PLS-DA from the mean-centered, TIC-normalized data sets (without autoscaling). Additionally, significance testing was performed on a MALDI matrix ion (m/z 273) for comparison. A one-tailed Student’s t-test was performed on the signal intensities at three significance levels (95%, 98%, and 99%). The results (Table 2-1) demonstrate a significant difference for many of the features identified by PCA and PLS-DA in the lysoPL region, including m/z 496, 518, 524, 544, and 546, each positively correlated with infarcted tissue. Additionally, two m/z values in the intact PL region, m/z 832 and 848, correlated negatively with infarcted tissue and passed the t-test at a significance level of 95% or greater. The opposite trends for these two lipid classes support increased PLA₂ activity in the infarcted myocardium.

Targeted MSⁿ studies for ion identification were conducted on significant features within the region of interest utilizing CID and PQD. MS² experiments revealed the major contribution of all significant features to be intact PC, intact PE, LPC, or LPE species. Previous work has demonstrated that MS² differentiates alkali metal adducts of PEs, alkali metal adducts of PCs, and protonated PCs; a neutral loss (NL) of 43 or 59 indicates the presence of an alkali metal adducts of a PE or PC, respectively⁹¹,⁹² whereas a fragment ion at m/z 184 indicates a protonated PC.³¹ Protonated PCs and LPCs yield relatively uninformative fragmentation; however, the mass of the fatty acid tail can be inferred for LPCs, as only one fatty acid tail is present. Fragmentation of alkali metal adducts of PCs and LPCs produces more relevant structural information.
MS³, fragmenting the daughter ion resulting from a NL of 59, was used to determine the presence of sodium or potassium in cationization. In this instance, a NL of 146 indicates the presence of a sodiated species, whereas a NL of 162 indicates the presence of a potassiated species. For LPCs, the remaining mass can be accounted for by the glycerol backbone, a hydroxyl group in the sn-2 position, and the lone fatty acid tail in the sn-1 position. Low abundance neutral losses corresponding to the mass of the fatty acid tails, which are crucial for structural elucidation of intact PCs containing two fatty acid tails, may also be observed in alkali cationized PC fragmentation. The tentative identification for all of the significant features is listed in Table 2-1. At m/z 520 and m/z 544, MS² revealed isobaric lipids, specifically an LPC and LPE at m/z 524 and two LPCs at m/z 544. Representative MS² spectra for m/z 520, 546, and 848 are shown in Figures 2-8, 2-9, and 2-10 respectively.

MS² analysis using CID averaged over cardiac tissue following LAD coronary artery ligation demonstrated a number isobaric lipids at m/z 520 (Figure 2-8). The most intense fragment ions occur at m/z 502 and 184, indicating the presence of a protonated LPC, specifically LPC 18:2. Since the unsaturated fatty acid tails are typically found at the sn-2 position of the glycerol backbone, it is unlikely that this LPC formed via the action of PLA₂. The next most intense fragments occur from NLs of 43 and 61 at m/z 477 and 459, respectively. These fragments would indicate the presence of a cationized PE at this nominal m/z, likely the [M+K]⁺ of LPE 18:0. Unlike LPC 18:2, LPE 18:0 is a likely end-product of hydrolysis by PLA₂, as the saturated fatty acid tails are primarily found at the sn-1 position of the glycerol backbone. Thus, one might anticipate that the major contribution of signal in infarcted tissue may arise from the LPE. Finally,
a NL of 59 is present. This NL could nominally result from an alkyl-linked LPC, more commonly known as a lysoplasmenylcholine (O-LPC). The structure of this ion is unidentified.

In contrast to m/z 520, m/z 546 produced fragments relating to a single abundant LPC. MS² analysis (Figure 2-9A) demonstrated an abundant NL of 59 (trimethylamine from the PC headgroup), indicating a cationized PC. MS³ of m/z 546→487 (Figure 2-9B) produced abundant NLs of 124 and 146 and an ion at m/z 147. The NL of 124 represents the fragmentation of the remainder of the PC headgroup, and the NL of 146 represents the loss of neutral sodiated cyclophosphane. If the NL occurs from the other half of the lipid, sodiated cyclophosphane manifests itself as an ion at m/z 147. These three fragment ions would indicate the presence of a sodiated LPC. From the remaining mass present in the lipid, m/z 546 was identified as the [M+Na]⁺ of LPC 18:0.

For intact PCs, PQD was used for compound identification in order to circumvent the low-mass cutoff (LMCO) normally present for CID experiments on the linear ion trap. For all PQD experiments, the LMCO was reduced to 50 Da. In a typical CID experiment isolating and fragmenting m/z 848, the LMCO, which is determined by the q isolation (0.25 on the Thermo LTQ XL), would have been 230 Da. This LMCO would preclude characteristic fragment ions for all protonated, sodiated, and potassiated ions (m/z 184, 147, and 163, respectively). An MS² spectrum utilizing PQD from cardiac tissue is shown for m/z 848 in Figure 2-10. In this instance, the dominant fragment occurs from a NL of 59, indicating a cationized PC. Additionally, fragment ions are observed from NLs of 183 and 221. The 38 Da difference between these two fragments would indicate the presence of potassium. Furthermore, the fragment at m/z 163 is
indicative of potassiated cyclophosphane. MS\(^3\) using CID of \(m/z\) 848→789 demonstrated a minor NL of 284, commonly corresponding to oleic acid. Therefore, the ion was identified as the \([M+K]^+\) of PC (18:0/20:4).

Mass spectrometric images were generated from infarcted cardiac sections for each of the identified LPCs and PCs. Representative images for three lysoPLs (Figure 2-11A, 2-11B, and 2-11C) and one intact PC (Figure 2-11D) are shown. For each LPC or LPE, an intense ion signal is observed in the core of infarcted myocardium, and an area of less intense ion signal is observed moving outward from this area. In contrast to the lysoPLs, pertinent intact PCs, such as PC (18:0/20:4), demonstrate the reverse trend. An area of relatively low signal is present near the core of infarcted myocardium, and an area of more intense signal is observed moving outward from this area, suggesting that intact PCs are being converted to LPCs via the action of PLA\(_2\) within the area of infarction.

To verify the major contribution to infarcted tissue of \(m/z\) values containing isobaric lipids, MS\(^2\) imaging experiments were conducted over cardiac tissue following LAD coronary artery ligation. An example of this strategy is shown as an inset in Figure 2-8. In this instance, \(m/z\) 184 was chosen as the characteristic fragment for LPC 18:2; \(m/z\) 502 (NL of 18) was not chosen as it is typically assigned as a loss of water, which is considered a non-specific neutral loss that can occur from a number of compounds naturally present in tissue or the MALDI matrix. For LPE 18:0, \(m/z\) 477 (NL of 43) was chosen as the characteristic fragment, although \(m/z\) 459 (NL 61) could have been chosen as well. The characteristic fragment for the LPE demonstrates a noticeably high ion signal in infarcted tissue relative to the LPC; thus, it was determined that the LPE is
the major contribution to the signal in infarcted tissue at \( m/z \) 520. This is an expected finding, as it was hypothesized that PLA\(_2\) would be more active in producing LPE 18:0 than LPC 18:2, as previously described in this chapter.

A systematic study was performed to determine the PLA\(_2\) activity as a function of the \( sn-2 \) fatty acid tail. In doing so, MS\(^2\) imaging experiments were performed for common intact PCs and PEs containing stearic acid (18:0) in the \( sn-1 \) position of the glycerol backbone. For all species, the partial head group loss (NL 43 for PEs and NL 59 for PCs) from the [M+Na]\(^+\) was monitored. A full list of the experiments performed and the ions monitored is detailed in Table 2-2. In all eight of the ions sampled, the fragment ion intensity decreased within the infarcted myocardium (Figures 2-12 and 2-13). Furthermore, both PCs and PEs demonstrated the same pattern in terms of % signal decrease; PLA\(_2\) demonstrated the greatest activity towards docosahexanoic acid, followed by arachidonic acid, oleic acid, and finally linoleic acid. Interestingly, these experiments also suggest that PLA\(_2\) may have a greater activity following MI towards intact PEs than PCs.

Multivariate data analysis and MS\(^n\) also shed light upon a number of sphingolipids and their localization following LAD coronary artery ligation. In particular, \( m/z \) 725 was identified by PCA as positively correlating with the infarcted myocardium. Based on targeted MS\(^n\) experiments, this ion was identified as a sphingomyelin, specifically the [M+Na]\(^+\) of SM(d18:1/16:0). In addition to \( m/z \) 725, \( m/z \) 264 demonstrated increased signal in infarcted myocardium. This ion at \( m/z \) 264 is hypothesized to be the d18:1 sphingosine base, which can be formed from enzymatic cleavage or source fragmentation from ceramides, sphingomyelins, or sphingosine-1-phosphate. Although
PCA and PLS-DA did not identify any ceramides as colocalizing with the d18:1 sphingosine base or sphingomyelins, the most intense fragment ion for ceramides containing the d18:1 sphingosine base (m/z 264) was observed in the MS\(^2\) spectrum of m/z 520. This fragment demonstrated localization in the infarcted myocardium, yielding a similar localization to both the LPCs and the LPEs. As discussed earlier, the primary ion at m/z 520 within infarcted myocardium was determined to be an LPE; however, the sphingosine fragment ion suggests the presence of a dehydrated ceramide containing both the d18:1 sphingosine base and palmitic acid, that is the [M+H-H\(_2\)O]\(^+\) of Cer (d18:1/16:0). In addition to the ceramide containing a palmitic acid tail, the most abundant ceramides in myocardium, as determined by Knapp et al., were all submitted to MS\(^2\) imaging. For these experiments, the [M+H-H\(_2\)O]\(^+\) was fragmented, and the fragment ion corresponding to the d18:1 sphingosine base at m/z 264 was mapped (Figure 2-14). In all instances, this fragment ion demonstrated localization in the infarcted myocardium, substantiating the previously determined localization of sphingomyelins and the sphingosine base. For illustrative purposes, an isobaric ion to Cer (d18:1/22:0) at m/z 604 demonstrating localization in the perfused myocardium is also shown.

In addition to the lipids discussed above, a number of other metabolites were found to be affected as a result of the LAD coronary artery ligation. PCA analysis demonstrated a number of low mass ions positively correlating with the perfused myocardium, including m/z 132, 162, and 348. Like the ion at m/z 132 (creatine), m/z 162 and 348 are hypothesized to be water-soluble metabolites that leak into the interstitial fluid following rupture of the plasma membrane. Targeted MS\(^2\) experiments
were conducted in perfused myocardium to identify these metabolites. Fragmentation of the ion at $m/z$ 348 demonstrated a single abundant ion at $m/z$ 136 (NL 212), corresponding to protonated adenine (Figure 2-15). Thus, the ion at $m/z$ 348 was identified as the $[M+H]^+$ of adenosine monophosphate (AMP). MS$^2$ analysis of $m/z$ 162 resulted in two fragment ions of similar abundance, $m/z$ 103 and $m/z$ 60, suggesting that $m/z$ 162 is the $[M+H]^+$ of carnitine (Figure 2-16). The fragment ion at $m/z$ 103 (NL 59) corresponds to a loss of trimethylamine, and the fragment ion at $m/z$ 60 (NL 102) corresponds to a loss of 3-hydroxybut-3-enoic acid.

**At-risk myocardium and perfused myocardium**

PCA was also conducted on an MSI dataset collected from cardiac tissue following LAD ligation to determine further markers for at-risk myocardium, in addition to TAGs. In doing so, representative mass spectra from at-risk myocardium and perfused myocardium were extracted as detailed above. The 2-dimensional scores plot constructed from PC 1 and PC 2 following PCA demonstrates separation between the two sample groupings (Figure 2-17), with PC 1 accounting for 61.4% of the variance. Furthermore, PC 1 dictated separation between at-risk and perfused myocardium. Analysis of the sample groupings demonstrated a tighter cluster for the perfused myocardium, suggesting a larger amount of biological variation within at-risk myocardium.

A 1-dimensional loadings plot was created for PC 1 to determine markers for at-risk myocardium (Figure 2-18). As expected, ions corresponding to TAGs (e.g., $m/z$ 879 and 903) positively correlated with the at-risk myocardium, and a number of intact PCs (e.g., $m/z$ 806, 820, and 848) positively correlated with perfused myocardium. Furthermore, carnitine at $m/z$ 162 and AMP at $m/z$ 348 also positively correlated with
perfused myocardium. A number of unexpected findings were gleaned from analysis of the loadings plot. Namely, three intact PCs, \( m/z \) 780, 808, and 832, were found to load with the at-risk tissue. All of these PCs were identified by MS\(^2\) as being sodium adducts of PCs that contain either linoleic acid \((m/z \) 780 and 808\) or arachidonic acid \((m/z \) 832\). Furthermore, the loadings plot demonstrated an ion at \( m/z \) 337, presumably a source fragment from linoleic acid containing lipids, also positively correlated with at-risk myocardium. Finally, the two TAGs with the highest loadings coefficients, \( m/z \) 879 and 903, were putatively identified as linoleic acid containing TAGs. Thus, linoleic acid may be associated with the tissue’s physiological response to myocardial ischemia.

**At-risk myocardium and infarcted myocardium**

PCA was also conducted on spectra extracted from the at-risk and infarcted myocardium. The scores plot, displayed in Figure 2-19, demonstrates separation between these two regions along the first principal component, which accounted for 77.2\% of the variance. The second principal component accounted for a 10-fold smaller amount of the variance (6.7\%) and largely accounted for the biological variation within the sample groupings, the majority of which occurred in the at-risk myocardium. A 1-dimensional loadings plot was then created to analyze the spectra features that drive the separation along the first principal component (Figure 2-20). Unfortunately, little new information was gleaned from this analysis as the separation was largely dictated by the differences between the lysoPL and intact PL content of the two tissue regions.

**PCA of all three regions of interest**

In addition to the PCA analysis conducted above, PCA was also performed to analyze all three regions (i.e., infarcted, perfused, and at-risk myocardium) in a single analysis. The scores plot from this analysis is displayed in Figure 2-21, in which the
three regions of myocardium are well separated on the first and second principal components. The first principal component demonstrated separation between the infarcted and perfused myocardium. This result was not unexpected, as the first principal component by definition carries the largest amount of variance (59.3% in this case), and the most significant histological and chemical differences are observed between these two regions. Accounting for 23.7% of the variance, the second principal component largely accounted for distinction between at-risk myocardium and perfused myocardium.

Although sufficient separation was achieved in the scores plot, the loadings plots were examined to determine if the markers determined by this analysis agree with the two-region comparisons discussed above. Initially, the loadings for the first principal component were examined (Figure 2-22). Similar to the two-region comparison, the majority of the variation on the first principal component was found in the intact PLs, mainly loading with perfused myocardium, and the lysoPLs, mainly loading with the infarcted myocardium. As exhibited in Figure 2-23, principal component 2 also yielded similar features for the two- and three-region comparisons. Most importantly, a number of TAGs loaded with the at-risk myocardium. Furthermore, various sodium adducts of intact PLs (m/z 766, 780, 790, 808, and 832) loaded with the at-risk myocardium, further suggesting that greater concentrations of sodium, and possibly linoleic acid, exist in the at-risk myocardium.

Comparing multiple biological samples

The feasibility of applying the developed multivariate data analysis method to independent biological samples was also investigated. To greatly minimize any variation that might occur in sample preparation, all biological samples were thaw-
mounted onto a single microscope slide. In the first attempt, a total of 3 tissues (2 infarct and 1 sham surgery) were analyzed. Upon availability of more biological specimens, the procedure was repeated with a total of 5 tissues (2 infarct and 3 sham surgeries) with similar results.

The results from the first comparison are shown in Figure 2-24. Similar to PCA of the infarcted and perfused myocardium from a single biological sample, separation between the infarcted myocardium and the perfused myocardium was observed on the first principal component. Furthermore, the sham surgery, labeled as “OR_Control” clustered with the perfused myocardium from the ligation surgeries. The tight clustering of the sham surgeries and perfused myocardium suggests that the perfused myocardium is either unaffected by the local blood deprivation in the left ventricle or that the chemical alterations occurring cannot be detected with this analysis. The second principal component, accounting for only 10.7% of the variance, dictates the sample-to-sample variation.

It should be noted that the previous analysis was conducted with the infarcted and perfused myocardium from ligation surgeries and control myocardium from sham surgeries; however, at-risk myocardium was omitted from the analysis. At-risk areas from the two ligation surgeries were subsequently added to further evaluate the potential to use this multivariate methodology on multiple biological samples. The results from this analysis are displayed in Figure 2-25. The inclusion of all three tissue regions and multiple biological samples served to complicate interpretation of the PCA scores plot; however, the first principal component (accounting for 58% of the variance) still dictated separation between the infarcted myocardium and the perfused
myocardium. The second principal component (accounting for 15.5% of the variance, which previously dictated separation between at-risk myocardium and perfused myocardium when only one biological sample was included, still demonstrates this separation, but also splits the biological samples for infarcted myocardium.

Accordingly, interpretation of the loadings plots is complicated by the splitting of the infarcted myocardium along principal component 2. Although a 1-dimensional loadings plot of principal component 1 (Figure 2-26) can be used in this scenario to determine localization for ions in the infarcted and perfused myocardium, a 1-dimensional loadings plot of principal component 2 (Figure 2-27) is insufficient to fully interpret ions localizing within the at-risk myocardium. In this instance, a 2-dimensional loadings plot (Figure 2-28A), or alternatively a biplot (Figure 2-28B), would be much more informative as the at-risk myocardium should positively correlate with ions that have negative loadings coefficients on principal component 1 and positive loadings coefficients on principal component 2. Regardless, this multivariate data analysis method has demonstrated the ability to distinguish condition from control despite the presence of biological variation.

**Unbiased multivariate data analysis and tandem mass spectrometry**

In addition to the methodology discussed above, an unbiased PCA approach was conducted similar to that conducted by van Hove et al.\(^5^0\) In this approach, each individual pixel from the dataset was deemed an independent sample (excluding pixels off tissue). Initially, spectral and spatial data were extracted from the using an in-house data converter. Following data extraction, peak picking and alignment were performed using an algorithm developed at the FOM Institute AMOLF to reduce the size of the dataset and shorten computing time. Then, the data was normalized to the TIC, mean-
centered, and normalized to the standard deviation of the sample means for unit variance. Following normalization, a first iteration of PCA was performed. PCA scores images were generated for the first 4 principal components. Invariably, the first principal component scores image demonstrated separation of pixels off tissue and pixels on tissue. Pixels that demonstrated a score higher than a user-defined threshold were then removed. It should be noted that this threshold is adjusted so as not to exclude pixels corresponding to areas on tissue. Finally, mass features corresponding to known matrix and background analytes were removed.

After the above data processing, a second iteration of PCA was performed on the remaining data. Initially, scores plots were generated for the first 20 principal components. Although the maximum number of principal components is equal to the number of variables, principal components with a low percentage of the variance rarely demonstrated relevant localization. To this point, the first 20 principal components appear to be a reasonable cutoff for datasets with approximately 1000 variables (i.e., 2% of the principal components). Once a suitable number of principal components were displayed, loadings plots were generated for principal components that demonstrated relevant localization in the regions of interest.

Upon analysis of cardiac tissue following coronary artery ligation, the first principal component demonstrated separation between infarcted and perfused myocardium and the third principal component demonstrated separation between the at-risk myocardium and the remainder of the tissue (Figure 2-29). Loadings plots from these principal components correlated well with the guided method (Figure 2-30). For example, the first principal component in both methodologies demonstrated high loadings coefficients
for intact PC and PEs correlating with the perfused myocardium, and similarly with LPCs and LPEs correlating with infarcted myocardium. Similarly, principal component 2 in the guided method and principal component 3 in the unbiased method displayed TAGs correlating with at-risk myocardium.

In addition to the three ion distributions discussed above, a fourth ion distribution was detected in the negative portion of principal component 19, despite the relatively low contribution (0.5%) to the total variance (Figure 2-31). This ion distribution, though occupying just a few pixels of the at-risk myocardium, demonstrates high loadings coefficients for ions at m/z 400, 424, 426, and 428 (Figure 2-31). Targeted and whole-tissue imaging MS² experiments were conducted to identify the structure of these ions. Initial analysis yielded fragment ions resulting from a neutral loss of 59, presumably the loss of trimethylamine, for m/z 400 and 428. Furthermore, a neutral loss of 161, attributed to the loss of free carnitine, was observed for all four ions as well. Thus, the four ions were identified as palmitoyl carnitine, linoleoyl carnitine, oleoyl carnitine, and stearoyl carnitine for m/z 400, 424, 426, and 428 respectively. The MS² spectrum, fragmentation pattern, and MS² images of two characteristic fragments for palmitoyl carnitine are shown in Figure 2-32 as an example. Interestingly, the two unsaturated acylcarnitines (linoleoyl and oleoyl) exhibit an abundant neutral loss of 179. Although this fragment presumably results from successive losses of free carnitine followed by water from the dehydrated fatty acid tail, it is currently unclear as to why unsaturated and saturated acylcarnitines exhibit different fragmentation patterns.

**Conclusions**

This research is the first presentation of MSI displaying potential markers for MI within intact cardiac tissue. First, creatine, a metabolite involved in the creatine kinase
metabolic pathway, demonstrated decreased ion signal within areas of infarcted myocardium, agreeing well with TTC-stained hearts following LAD coronary artery ligation. The spatial localization of creatine proved to be a valuable indicator for infarcted myocardium; however, creatine did not distinguish between the affected and at-risk regions of tissue.

Following the targeted study for creatine, a combination of multivariate data analysis and tandem mass spectrometry was conducted on an MSI data set to identify PC, LPC, and LPE markers of MI. These studies indicated that many LPCs and select LPEs demonstrated increased ion signal, and conversely, select intact PCs and PEs demonstrated decreased ion signal within the area of infarction. Presumably, the lysoPLs are being formed via enzymatic hydrolysis by PLA₂. It is also interesting that two intact PCs found to decrease in infarcted tissue presumably contain arachidonic acid in the sn-2 position, as it has been reported that PLA₂ and arachidonic acid may play a role in the cellular response to myocardial infarction, specifically involving protection against ischemic cell death. The complementary localization of LPC 18:0 at m/z 546 and its precursor, PC (18:0/20:4) at m/z 848, support this hypothesis. Further research was conducted concerning the fatty acid specificity of PLA₂, and it was concluded that for stearic acid containing PLs, PLA₂ appears the most active for PLs containing docosahexanoic acid followed by arachidonic acid. This trend was observed using targeted MS² experiments for intact PCs and PEs. The PL findings of this study suggest that PLA₂ activity increased in infarcted myocardium following LAD ligation.

In addition to the glycerophospholipids discussed above, a number of sphingolipids were found to localize within the infarcted myocardium. Specifically, the
d18:1 sphingosine base, SM (d18:1/16:0), and nearly all of the abundant ceramides were found to localize in areas of necrosis. At present, the reason for the localization of sphingomyelin is unclear; however, arachidonic acid liberated by PLA$_2$ during ischemia has been hypothesized to stimulate sphingomyelinase (SMase) activity.$^{96,97}$ SMase is an enzyme that serves to hydrolyze intact sphingolmyelins, forming ceramides,$^{98}$ possibly offering an explanation for the increased content of ceramides in infarcted myocardium. Furthermore, Nakane et al. has speculated that an increase in both gangliosides and sphingosine accompany this increase in ceramide concentration following ischemia.$^{96}$ Alternatively, the d18:1 sphingosine base may result from fragmentation of the dehydrated ceramides within the MALDI ionization source.

The at-risk myocardium, which provides the most interesting biological relevance concerning MI, was also characterized using targeted tandem MSI and multivariate data analysis techniques. This tissue type, although considered ischemic, has the potential to become viable if reperfusion (i.e., the restoration of blood flow) is performed, thereby decreasing the overall size of the infarction and limiting cell death. The targeted tandem MSI studies demonstrated that TAGs were localized within the at-risk areas of infarcted hearts following LAD ligation. Although the current spatial resolution of this method does not permit a direct comparison of microscopic and MSI images, it is believed that lipid droplets are responsible for TAG localization in at-risk zones. Additionally, a number of linoleic acid containing intact PLs and TAGs were found to be positively correlated with the at-risk myocardium following PCA. Finally, a number of acylcarnitines were found to localize in limited areas of the at-risk myocardium. Previous research has suggested that acylcarnitines accumulate during acute
myocardial ischemia, demonstrating approximately a 10-fold increase in extracts following a rabbit model of ischemia. This study indicated that the likely cause of this accumulation of acylcarnitines was inhibition of fatty acid oxidation in the mitochondria. As inhibition of fatty acid oxidation was also implicated in the accumulation of TAGs following MI, it is not surprising that TAGs and acylcarnitines colocalize in the at-risk myocardium. The implication of these findings is that inhibition of fatty acid oxidation may play a key role in myocardial protection under ischemic conditions.

It should be noted that tissue-specific markers do not immediately result in blood or plasma markers of MI. Nevertheless, it may be beneficial to investigate these tissue-specific markers in biological fluids using targeted, sensitive methods (e.g., selected reaction monitoring with triple quadrupole MS coupled to liquid chromatography). In particular, small metabolites such as creatine, free carnitine, and AMP that may leak into the interstitial fluid following infarction may demonstrate an increase in blood-borne concentration. The discovery of one or a number of small molecule markers would offer a valuable alternative to the traditional protein markers utilized in a clinical setting.

MALDI MSI offers advantages over traditional myocardial infarction characterization techniques such as histological staining and biological assays. In particular, the ability to detect, identify, and localize small molecular species allows for identification of putative markers for MI with a single analytical technique. Furthermore, a wealth of data was collected relatively quickly (e.g., approximately one hour for a single cardiac section), making these experiments prime for multivariate data analysis techniques commonly used in the ‘omics’ platforms. This research has presented two
PCA methodologies (guided and unbiased). When used in a complementary fashion, these techniques provide an extremely powerful basis for unprecedented exploratory MSI research. Thus, MALDI MSI, coupled with multivariate data analysis has the potential to be applied to a wide variety of tissue applications, making it a powerful tool for diseased-state characterization and biomarker discovery.
Figure 2-1. MSI analysis of cardiac tissue following ligation surgery. A) Enzymatic reduction of TTC to TPF, B) Photograph of the upper half of a heart stained with TTC following LAD coronary artery ligation. Unstained tissue, outlined in blue, indicates tissue damage from myocardial necrosis. C) MS image of m/z 132 normalized to the TIC and D) MS² image of m/z 132 → 90 from the lower half of the same heart depicted in B. Lower ion signal was observed in the infarcted region near the right side of the tissue section.
Figure 2-2. MS image of $m/z$ 132 normalized to the TIC from control cardiac tissue.
Figure 2-3. Mass spectra of the lipid region collected in different regions of myocardium. Spectra were acquired from either A) infarcted or B) perfused zones of cardiac tissue.
Figure 2-4. MALDI MS spectra acquired from sham surgery myocardium. The spectra were acquired from locations with either A) in vitro digestion by PLA$_2$ or B) no enzymatic alteration.
Figure 2-5. Example of TAG identification and localization. A) MS\(^2\) spectrum of \(m/z\) 881 from the at-risk region of cardiac tissue following LAD coronary artery ligation and MS\(^2\) images of B) \(m/z\) 881→599 and C) 881→625 from the same tissue section. The primary ion at \(m/z\) 881 is TAG 52:2.
Figure 2-6. Multivariate data analysis of infarcted and perfused myocardium. A) PCA scores plot and B) PLS-DA scores plot for mean-centered, TIC-normalized data. Each sample consists of 25 spectra taken along a horizontal line within either infarcted (green crosses) or healthy tissue (red triangles) from a single tissue section following an LAD coronary artery ligation. The ovals indicate the 95% confidence interval for the sample groupings.
Figure 2-7. PCA loadings plots dictating separation between infarcted and perfused myocardium. The data were processed using 2-methods: either A) mean-centered data or B) autoscaled data.
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<th>MS^n Identification</th>
<th>Ion</th>
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Figure 2-8. MS$^2$ spectrum using CID of $m/z$ 520 from infarcted cardiac tissue. Fragment ions are identified from two isobaric lipid ions: the [M+K]$^+$ of LPE 18:0 (squares) and the [M+H]$^+$ of LPC 18:2 (stars). Furthermore, the structures of each lipid and MS2 images of each species’ characteristic fragment (i.e., $m/z$ 520→477 for LPE 18:0 and $m/z$ 520→184 for LPC 18:2) from cardiac tissue following LAD coronary artery ligation are shown as an inset. The LPE contributes more ion signal within the infarcted zone than the LPC.
Figure 2-9. Example of MS\textsuperscript{n} identification and imaging of an LPC. A) MS\textsuperscript{2} spectrum using CID of \textit{m/z} 546 and B) MS\textsuperscript{3} spectrum using CID of \textit{m/z} 546 → 487 from infarcted cardiac tissue. The major fragment in A is a NL of 59, indicating a cationized PC. Furthermore, in B, the 22 Da difference between \textit{m/z} 363 and 341 indicate a sodiated PC. Also, the fragment ion at \textit{m/z} 147 is indicative of sodiated cyclophosphane, a characteristic ion of sodiated PCs. The ion was identified as the [M+Na]\textsuperscript{+} of LPC 18:0. The structure and an MS\textsuperscript{2} image of \textit{m/z} 546 → 487 from cardiac tissue following LAD coronary artery ligation is shown as an inset in A.
Figure 2-10. MS² spectrum of $m/z$ 848 using PQD from infarcted cardiac tissue. A NL of 59 indicates a cationized PC. The 38 Da difference between $m/z$ 655 and 627 and the fragment ion at $m/z$ 163 (potassiated cyclophosphane) indicate a potassiated PC. MS³ demonstrated a minor NL of 284, indicating a stearic fatty acid tail in the $sn$-1 position of the glycerol backbone (data not shown). Therefore, the ion was identified as the [M+K]$^+$ of PC (18:0/20:4). The structure and an MS² image of $m/z$ 848 $\rightarrow$ 789 from cardiac tissue following LAD ligation are shown as an inset.
Figure 2-11. MS images normalized to the TIC from a heart following LAD coronary artery ligation. A) the [M+K]$^+$ of LPE 18:0 at $m/z$ 520, B) the [M+Na]$^+$ of LPC 18:0 at $m/z$ 546, C) the [M+Na]$^+$ of LPC 16:0 at $m/z$ 518, and D) the [M+K]$^+$ of PC (18:0/20:4) at $m/z$ 848.
Table 2-2. MS$^2$ imaging experiments performed ion the [M+Na]$^+$ ions from intact PC and PE ions containing stearic acid in the sn-1 position of the glycerol backbone.

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Figure 2-12. MS² PC fragment ion intensity (loss of trimethylamine) for four common intact PCs in infarcted (blue) and perfused (red) myocardium. All four of these ions contain stearic acid in the sn-1 position. Error bars represent the standard deviation of the mean (n=30).
Figure 2-13. MS² PE fragment ion intensity (loss of ethenamine) for four common intact PEs in infarcted (blue) and perfused (red) myocardium. All four ions contain stearic acid in the sn-1 position, but differ in sn-2 fatty acid tail. Error bars represent the standard deviation of the mean (n=30).
Figure 2-14. MS$^2$ images of select abundant ceramides in cardiac tissue following coronary artery ligation. All MS$^2$ experiments were performed on the [M+H-H$_2$O]$^+$. For contrast, an unidentified ion that demonstrates complementary localization and is nominally isobaric to the ceramide at m/z 604 is also displayed.
Figure 2-15. MALDI MS\(^2\) spectrum of m/z 348 obtained from perfused myocardium following LAD ligation surgery. The MS image (right) and fragmentation pattern of AMP (center), which is proposed as the dominant species at m/z 348, is also displayed.
Figure 2-16. MALDI MS$^2$ spectrum of $m/z$ 162 obtained from perfused myocardium following LAD ligation surgery. The MS image (top) and fragmentation pattern of free carnitine (bottom), which is proposed as the dominant species at $m/z$ 162, is also displayed.
Figure 2-17. PCA scores plot demonstrating the separation between at-risk (red) and perfused (green) myocardium. Each sample represents an average of approximately 10 mass spectra.
Figure 2-18. Principal component 1 loadings plot dictating the separation between perfused and at-risk myocardium. Additionally, the MS images of two ions, indicative of the two tissue regions, are displayed. The ion at m/z 348, identified as AMP is indicative of perfused myocardium, and the ion at m/z 879, identified as TAG (16:0/18:1/18:2), is indicative of the at-risk myocardium.
Figure 2-19. PCA scores plot demonstrating the separation between at-risk (red) and infarcted (green) myocardium. Each sample represents an average of approximately 10 mass spectra.
Figure 2-20. Principal component 1 loadings plot dictating the separation between perfused and infarcted myocardium.
Figure 2-21. PCA scores plot generated from sampling the infarcted (green), perfused (blue), and at-risk myocardium (red) following coronary artery ligation. The ellipses represent the 95% confidence interval of the sample groupings.
Figure 2-22. Principal component 1 loadings plot following PCA analysis of all three regions of myocardium. This principal component, accounting for 59.3% of the variance, dictates separation between the infarcted (positive portion) and perfused myocardium (negative portion).
Figure 2-23. Principal component 2 loadings plot following PCA analysis of all three regions of myocardium. This principal component, accounting for 23.7% of the variance, dictates separation between the at-risk (positive portion) and perfused myocardium (negative portion).
Figure 2-24. PCA scores plot generated from analysis of three biological samples. Principal component one demonstrates separation between infarcted myocardium from ligation surgeries (negative portion) and perfused myocardium from both sham and ligation surgeries (positive portion).
Figure 2-25. PCA scores plot generated from analysis of multiple biological samples. Furthermore, all three tissue regions (infarcted, perfused, and at-risk myocardium) were included in the analysis.
Figure 2-26. Loadings plot of principal component 1 generated from PCA of multiple biological samples including three different regions of myocardium. This principal component largely dictates the separation between infarcted myocardium and the remaining tissue regions.
Figure 2-27. Loadings plot of principal component 2 generated from PCA of multiple biological samples including three different regions of myocardium.
Figure 2-28. Graphical representation of loadings from PCA of multiple biological samples with the inclusion of all three tissue regions. Two different methods are displayed: A) a 2-dimensional loadings plot and B) a biplot.
Figure 2-29. Principal component scores images from unbiased PCA analysis. Principal component 1, accounting for 13.2% of the total variance, demonstrates separation between the perfused (negative) and infarcted myocardium (positive). Principal component 3, accounting for 5.3% of the total variance, demonstrates separation between the at-risk (negative) and perfused myocardium (positive).
Figure 2-30. Loadings plots from unbiased PCA analysis. Principal component 1 (top) demonstrates separation between infarcted and perfused myocardium and principal component 3 (bottom) demonstrates separation between perfused and at-risk myocardium.
Figure 2-31. Loadings plot of principal component 19 (PC 19) from unbiased PCA analysis. The scores images for the negative portions of PC 19 and principal component 3 (PC 3) are also displayed.
Figure 2-32. MS$^2$ spectrum of m/z 400 collected from cardiac tissue following experimental coronary artery ligation surgery. The fragmentation pattern of the identified ion (palmitoyl carnitine) and MS$^2$ images of two characteristic ions displaying increased signal in the at-risk myocardium are also displayed.
CHAPTER 3
DEVELOPMENT OF 9-AMINOACRIDINE AS A DUAL-MODE MALDI MATRIX FOR SMALL MOLECULE MASS SPECTROMETRIC IMAGING STUDIES OF MYOCARDIAL INFARCTION

Introduction

Matrix-assisted laser desorption/ionization (MALDI) is a soft ionization mechanism suitable for the analysis of small and large biomolecules. Since the two breakthrough MALDI MS publications by Tanaka et al.\textsuperscript{5} and Karas et al.,\textsuperscript{3} a number of small organic compounds (matrices) have become well established for the analysis of lipids,\textsuperscript{90,99} proteins,\textsuperscript{1,3,100} and peptides\textsuperscript{101} in positive ionization mode. In general, this group of compounds consists of weak acids that exhibit strong absorption in the ultraviolet portion of the electromagnetic spectrum, affording the ability to protonate analytes to form positive ions. As MALDI is still a relatively young ionization method, an all-purpose negative mode matrix has primarily eluded researchers.

Recently, a number of small organic compounds have been proposed as potential negative mode MALDI matrices. As opposed to the positive mode matrices, which typically exhibit weak acidity via functional groups such as carboxylic acid, these compounds are generally basic in nature. The most promising set of these potential matrices are aromatic compounds containing at least one amino group. The structures of three of these potential matrices, 9-aminoacridine (9-AA), 1,5-diaminonaphthalene (DAN), and 1,8-bis(dimethylamino)naphthalene (DMAN), are displayed in Figure 3-1. Of these three matrices, DMAN, also known as a “proton sponge”, is the most basic, exhibiting a \( pK_b \) of approximately 1.9.\textsuperscript{102} Although DMAN exhibits ‘superbasic’ properties, recent research has revealed that DMAN produces an unstable signal under the high vacuum source conditions employed by most MALDI MS instruments.\textsuperscript{103,104}
This signal instability is likely a result of the relatively high volatility of DMAN, causing matrix crystals to sublime from the target surface over time. DAN is a relatively weak base (pK_b~9.56), and has recently gained interest in the MSI community.\textsuperscript{103} This matrix was first reported by Catherine Costello’s group in 1992 for the analysis of gangliosides.\textsuperscript{105} Although there have been no reports of problems with signal stability, the relatively weak basicity of this matrix raises concerns about the breadth of analytes that can be analyzed in negative mode.

The most promising of these three candidates, 9-AA, is a fairly strong base (pK_b~4), and was first reported as a suitable negative-mode MALDI matrix by Vermillion-Salsbury et al. in 2002.\textsuperscript{106} Since this initial study, 9-AA has been reported as an effective negative-mode MALDI matrix for low molecular weight acids,\textsuperscript{107} endogenous metabolites,\textsuperscript{108, 109} and a variety of lipid classes.\textsuperscript{109-111} There has yet to be a report describing signal stability issues under high vacuum, and the basicity appears to be suitable to ionize analytes of interest that are ineffectively ionized in positive mode by acidic matrices such as DHB. Furthermore, there have been a number of publications describing the use of 9-AA as a matrix for MALDI metabolomics\textsuperscript{108, 109} and MALDI mass spectrometric imaging (MSI) applications.\textsuperscript{111-113}

Previous research has demonstrated the benefits of multivariate data analysis, namely principal component analysis (PCA), for the analysis of MALDI MSI datasets collected in positive mode with DHB.\textsuperscript{48} The present research utilized a rat model for myocardial infarction (MI), simulating a heart attack resulting in significant tissue death. This model, known as a left anterior descending (LAD) coronary artery ligation, deprives oxygenated blood to the left ventricular myocardium, leaving the right ventricular
myocardium largely unaffected. The result of this ligation model is that both the injured and control samples can be interrogated within a single tissue section. Using MSI and PCA, a number of tissue-specific alterations in lipid and metabolite profiles were observed. Although this endeavor interrogated a number of compound classes in positive mode, a number of analytes were excluded from the study due to inefficient ionization in positive mode. This chapter will demonstrate the utility of 9-AA as a suitable dual-mode MALDI matrix (positive and negative mode) for the analysis of MI with MSI and PCA.

**Experimental**

**Chemicals and reagents**

HPLC-grade water (H$_2$O) and HPLC-grade methanol (MeOH) were purchased from Fisher Scientific (Fair Lawn, NJ). 100% ethanol (EtOH) was purchased from Decon Labs (King of Prussia, PA). 9-aminoacridine (9-AA) and 9-aminoacridine hydrochloride hemihydrate were purchased from MP Biomedicals (Solon, OH). 2,5-dihydroxybenzoic acid (DHB) and β-nicotinamide adenine dinucleotide, reduced (NADH) were purchased from Sigma-Aldrich (St. Louis, MO). β-nicotinamide adenine dinucleotide in the non-reduced form (NAD$^+$) was purchased from Acros Organics (Geel, Belgium). Lipid standards, namely 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC 17:0), 1-hexadecanoyl-2-(9Z,12Z-octadecadienoyl)-sn-glycero-3-phosphoethanolamine (PE (16:0/18:2)), L-α-phosphatidylserine from porcine brain, and L-α-phosphatidylinositol from bovine liver were purchased from Avanti Polar Lipids (Alabaster, AL). 9-AA was dissolved in 70:30 EtOH:H$_2$O (v/v) to a final concentration of 6 mg/mL to serve as a MALDI matrix. For comparison, DHB was dissolved in 70:30 MeOH:H$_2$O (v/v) to a final concentration of 40 mg/mL. NAD$^+$ and NADH were dissolved
in H$_2$O and 0.01M NaOH, respectively, to a final concentration of 1 mg/mL to serve as nucleotide standards. The lipid standards were either dissolved (in the case of a powder) or diluted (in the case of a solution) to 1 mg/mL in EtOH for all standard analyses.

**Extraction of 9-AA free base**

As the free base of 9-AA is relatively expensive (~1g for $70) and the hydrochloride salt is much less so (~50g for $70), an acid-base extraction was performed, similar to that previously reported.$^{107}$ Approximately 400 mg of the hydrochloride salt was dissolved in 25 mL of boiling H$_2$O. Following removal from heat, sodium carbonate was added for alkalinization. The free base was then extracted with CHCl$_3$. The CHCl$_3$ layer was then obtained and the solvent was evaporated. The resulting solid was reconstituted in 70:30 EtOH:H$_2$O (v/v). The suspension was centrifuged, and the supernatant was utilized as a MALDI matrix.

**UV/Vis absorption spectrophotometry**

All experiments were conducted on a double beam Hewlett-Packard 8450A UV/Vis spectrophotometer equipped with a diode-array detector. Matched quartz cuvettes were used for the blank and sample solutions. Blank solutions were prepared according to the respective solvent systems for the MALDI matrices: 70:30 EtOH:H$_2$O (v/v) for 9-AA and 70:30 MeOH:H$_2$O for DHB. The wavelength range on the instrument was set to 200–900 nm. Prior to analysis of each sample, the baseline (or balance) was measured by analyzing the blank solution in both the sample and reference cuvettes. The blank solution in the reference cuvette was then replaced with the MALDI matrix, and UV/Vis absorption spectra were obtained.
Biological sample preparation

All animal procedures were conducted in accordance with guidelines published in the Guide for the Care and Use of Laboratory Animals (National Research Council, National Academy Press, Washington, DC, 2010) and were approved by the Animal Care Committee of Saint Louis University. A 24h LAD ligation (6 animals) was performed as previously described to induce myocardial ischemia. In addition to the ligation surgery case group, two separate control groups, sham (6 animals) and naïve (3 animals), were prepared. For the sham control, all surgical steps were conducted for the LAD ligation, except the LAD suture was tied loosely around the artery, retaining normal blood flow to the left ventricle. For the naïve control, no surgery was performed prior to euthanization. 24 hours following either LAD ligation or sham surgery, the rats were euthanized with pentobarbital (~800 mg/kg, i.p.). The hearts were then excised, flash-frozen in liquid nitrogen, and stored at −80 °C until further use.

Hearts from all sample groupings (infarction, sham, and naïve) were bisected along a transverse plane at the midventricular level. The upper half of the heart was subjected to 2,3,5-triphenyltetrazolium chloride (TTC) staining as previously described. The lower half of the heart was sectioned on a Microm HM 505E cryostat (Waldorf, Germany) at −25 °C to 10-μm thickness along the same transverse plane as the bisection. Following sectioning, tissues were thaw-mounted atop a microscope slide. In order to ensure the ability for efficient tissue-to-tissue comparison, tissues from different sample groupings were thaw-mounted atop the same microscope slide. Furthermore, serial sections were obtained on two separate microscope slides to compare the two prepared MALDI matrices (9-AA and DHB). Tissue sections were then stored at −80 °C until further use.
Prior to matrix application, tissues were dehydrated in a vacuum desiccator for approximately 45 minutes. Immediately following desiccation, tissue sections were coated with MALDI matrix (9-AA or DHB) using a Type A3 Glass Meinhard Nebulizer (Golden, CO). Care was taken to coat serial sections with 9-AA and DHB for the best comparison. Approximately 150 mg 9-AA (25 mL of solution) or 400 mg DHB (10 mL of solution) was deposited atop the microscope slide before adequate crystallization was observed atop the tissue sections. It should be noted that excessive tissue wetting was avoided to minimize analyte migration.

**Mass spectrometry and imaging**

All experiments were performed on a Thermo Scientific LTQ XL (San Jose, CA) equipped with a Thermo MALDI ionization source. The MALDI ionization source consisted of an N2 laser (\(\lambda=337 \text{ nm}\)) with a repetition rate of 60 Hz and an observed laser spot diameter of approximately 100 \(\mu\text{m}\). The source region of the LTQ was maintained at intermediate pressure (~75 mTorr) for all imaging experiments unless otherwise noted. For all experiments with 9-AA in negative ionization mode, a laser energy of 7.5 \(\mu\text{J}\) and 3 laser shots per laser stop were utilized. For all experiments with DHB and 9-AA in positive ionization mode, a laser energy of 4 \(\mu\text{J}\) and 3 laser shots per laser stop were utilized. To preserve the number of laser shots per laser stop, automatic gain control (AGC) was toggled off. MS\(^2\) experiments were conducted with an isolation width of 1.2 amu and a collision energy between 30 and 45 A.U. A raster step size of 150 \(\mu\text{m}\) was utilized for all imaging experiments, unless otherwise noted.

**Statistical analysis**

Following MS imaging, PCA was conducted to determine tissue-specific markers of MI. Briefly, representative samples from each region of tissue were extracted from
the centroided imaging dataset. Each sample consisted of 5–15 spectra averaged from a horizontal line within the region of interest. Each individual sample was saved as a .CSV file and all of the samples were grouped according to the region of interest. The resulting groups were then compressed into a .ZIP file and imported into the Metaboanalyst Web Server. Once imported, a mass tolerance of 0.75 amu was utilized to account for any mass shifts due to space-charge effects. Major matrix peaks, resulting from either 9-AA or DHB were then removed. These matrix peaks were determined by manual inspection of regions surrounding the tissue sections. All intensities were then normalized by the total ion current (TIC). The data were then mean-centered or alternatively autoscaled for each m/z value. PCA was then performed, and 2-dimensional scores plots were generated to visualize the sample groupings in principal component space. Depending on the principal component that dictated the separation between sample groupings, a 1-dimensional loadings plot was generated to determine m/z values of interest.

Results

Standard characterization

Prior to use with cardiac tissue following coronary artery ligation, a number of standards were characterized using 9-AA as a negative mode MALDI matrix. Initial experiments examined two previously published solvent systems for 9-AA: 1) 60:40 IPA:ACN (v/v) and 2) 70:30 EtOH:H₂O (v/v). 9-AA proved to be fully soluble in the first solvent system at a concentration of 10 mg/mL. The same concentration prepared in the second solvent system generated a suspension. The suspension prepared in the second solvent system was spun down using a centrifuge, and the supernatant was collected to serve as the MALDI matrix. Both matrices were spotted on a 384-well
MALDI sample plate for characterization. Upon visual inspection of the spotted matrices, 9-AA prepared in solvent system 2 provided more homogenous crystallization than the first solvent system. It was later determined that the solubility of 9-AA was approximately 6–7 mg/mL in the second solvent system. Consequently, a MALDI matrix of 6 mg/mL 9-AA in 70:30 EtOH:H₂O (v/v) was used for standard characterization and tissue analysis.

**Phosphatidylcholine (PC)**

Previous experiments with MALDI-ToF MS at low source pressure produced [M-CH₃]⁻ ions from PCs utilizing 9-AA as a MALDI matrix. To determine if these ions are produced at intermediate pressure, such as the source pressure on a Thermo MALDI LTQ XL, a 1 mg/mL lysophosphatidylcholine (LPC) standard was prepared. The prepared standard, LPC 17:0 (MW=509 Da), was mixed in a 1:1 ratio with the MALDI matrix, and spotted using the traditional dried droplet method. MS analysis in negative mode produced the same ions previously reported, with the [M-CH₃]⁻ occurring at m/z 494. MS² was then conducted to characterize the fragmentation pattern of this ion. The MS² spectrum of m/z 494 yielded an abundant ion at m/z 269, corresponding to the [M-H]⁻ of heptadecanoic acid (Figure 3-2). Less abundant ions were observed at m/z 224 and 242 corresponding to the [M-CH₃-H₂O]⁻ and [M-CH₃]⁻, respectively, of glycerophosphocholine.

The same matrix/analyte spot was also analyzed in positive mode. Abundant ions were observed at m/z 510, corresponding to the [M+H]⁺ and, m/z 1019, corresponding to the [2M+H]⁺ of LPC 17:0. Although abundant protonated monomer and dimer ions were observed, no appreciable alkali metal adducts were observed in the positive mode analysis of this lipid standard with 9-AA. MS² fragmentation of the protonated monomer
at m/z 510 yielded the typical PC fragment at m/z 184, corresponding to the phosphocholine head group. Fragmentation of the protonated dimer at m/z 1019 yielded ions at m/z 510 (the protonated monomer), m/z 496 (the demethylated protonated monomer), and m/z 524 (the methylated protonated monomer).

**Phosphatidylethanolamine (PE)**

A major disadvantage reported in the literature for 9-AA as a negative mode MALDI matrix is the inability to distinguish PCs and PEs. The characteristic ion of a PC in negative mode, the [M-CH$_3$]$^-$, and the characteristic ion of a PE with two less carbons contained within the fatty acid substituents, the [M-H]$^-$, are isomeric. Although this is a problem for instruments with only one stage of mass analysis, tandem MS can differentiate these isomers.

A 1 mg/mL synthetic standard of PE (16:0/18:2) (MW = 715 Da) was mixed in a 1:1 ratio with the prepared 9-AA matrix solution. The mixture was then spotted onto a MALDI target plate and allowed to dry with the aid of gentle heating. Negative mode MALDI MS analysis of the spotted analyte/matrix mixture yielded an ion at m/z 714, corresponding to the [M-H]$^-$. CID of m/z 714 (Figure 3-3) yields a number of abundant fragment ions, with the two most abundant fragment ions, m/z 279 and 255, corresponding to the carboxylate anions of linoleic acid (18:2) and palmitic acid (16:0), respectively. Furthermore, the ions at m/z 452 and 476 correspond to the loss of dehydrated linoleic acid and palmitic acid, respectively. Once the two fatty acid tails are identified, the remainder of the mass can be attributed to the glycerol back bone and the head group. In contrast, for the isomeric PC (18:0/18:2), one would expect fragment ions at m/z 279 and 284, corresponding to the carboxylate anions of linoleic acid (18:2) and stearic acid (18:0), respectively.
Phosphatidylserine (PS)

Further characterization of lipid standards using 9-AA as a MALDI matrix in negative mode was performed on an l-α-phosphatidylserine (PS) extract from porcine brain. MS analysis of this extract produced two abundant ions at \( m/z \) 701 and 788 (Figure 3-4). Furthermore, low abundant ions were observed at \( m/z \) 747 and 834. Interestingly, both pairs of ions were separated by 87 amu. The lower mass ion in both pairs of ions is thought to be a source fragment resulting from the loss of serine. Furthermore, the intensity ratio of \( m/z \) 701 to 788 and \( m/z \) 747 to 834 are similar, suggesting that the degree of source fragmentation is maintained for both species.

The suspected intact PS ions were then subjected to MS\(^2\) analysis, and the fragmentation pattern for PS ions in negative mode was determined. The MS\(^2\) spectrum of \( m/z \) 788 is displayed in Figure 3-4A. Unlike the other phospholipids examined, PS ions in negative mode exhibit a neutral loss related to the head group. More specifically, this neutral loss results from the cleavage of serine (NL 87), further suggesting that the ions at \( m/z \) 701 and 747 in the MS spectrum are source fragments. MS\(^3\) of \( m/z \) 788→701 demonstrates fragmentation related to the fatty acid tails (Figure 3-4B). The ions at \( m/z \) 283 and 281 correspond to the sn-1 fatty acid tails of stearic acid (18:0) and oleic acid (18:1), respectively, identifying \( m/z \) 788 as the [M-H]\(^-\) of PS (18:0/18:1). Additionally, neutral losses on both sides of the ester linkage to the glycerol backbone are observed for both of these tails. Similar MS\(^2\) fragmentation was observed for \( m/z \) 834; however, MS\(^3\) fragmentation of \( m/z \) 834→747 suggested fatty acid tails of stearic acid (18:0) and docosahexanoic acid (22:6), identifying the precursor ion as the [M-H]\(^-\) of PS (18:0/22:6). Thus, when one considers the PS fragmentation pathway, the
MS\(^2\) spectrum confirms the presence of a PS, and MS\(^3\) yields information of the fatty acids bound to the glycerol backbone.

**Phosphatidylinositol (PI)**

An extract of l-\(\alpha\)-phosphatidylinositol (PI) from bovine liver was also analyzed in the manner discussed above. PIs have been shown to readily ionize in negative mode with a variety of MALDI matrices, including 9-AA.\(^{111,115}\) Furthermore, the negative mode fragmentation pathways of PIs have been reported in depth;\(^{116}\) therefore, the results of this study will only be briefly reported in this section. Analysis of the extract produced an abundant ion at \(m/z\) 863. Additionally, a number of low intensity ions were observed throughout the lipid region of the mass spectrum. Similar to PEs, MS\(^2\) fragmentation of these ions produced abundant carboxylate anions indicative of the fatty acid substituents esterified to the glycerol backbone. For instance, MS\(^2\) analysis of the precursor ion at \(m/z\) 863, yielded ions at \(m/z\) 283 and 281, corresponding to the [M-H]\(^-\) for stearic and oleic acid, respectively. Additionally, abundant neutral losses of the same fatty acids were observed at \(m/z\) 581 and 579.

**NAD\(^+\) and NADH**

In addition to negatively charged phospholipids, 9-AA has been reported to effectively ionize nucleotide species containing at least one phosphate group.\(^{108,109}\) Prior to tissue analysis, nicotinamide adenine dinucleotide (NADH), a water-soluble nucleotide that has previously been observed in myocardial extracts,\(^{109}\) was analyzed using the protocol discussed above. MS analysis of the spotted standard demonstrated an abundant ion at \(m/z\) 664, corresponding to the [M-H]\(^-\) of NADH (Figure 3-5). Following MS analysis, CID was conducted on this ion to characterize the fragmentation pattern of NADH (Figure 3-6). The most abundant ions resulted from cleavages along
the two phosphate functional groups bridging nicotinamide and adenine; the ions at \( m/z \) 408 and 397 result from cleavage of nicotinamide-\( \beta \)-ribose and adenosine, respectively. Additionally, cleavages are observed between the two phosphate groups, resulting in NLs of \( \beta \)-nicotinamide ribose monophosphate and adenosine monophosphate at \( m/z \) 346 and 335, respectively. Returning to the MS spectrum in Figure 3-5, the second most abundant peak that doesn’t correspond to a matrix ion is \( m/z \) 540. As this ion does not appear in the \( \text{MS}^2 \) spectrum of \( m/z \) 664, it is unlikely that \( m/z \) 540 is a source fragment. In contrast, ions corresponding to the most abundant NLs in the \( \text{MS}^2 \) spectrum of \( m/z \) 664 (e.g., \( m/z \) 408 and 346) are observed, and are hypothesized to result from source fragmentation.

In addition to the reduced form of nicotinamide adenine dinucleotide, the oxidized form of the same dinucleotide (NAD\(^+\)) was also characterized utilizing 9-AA as a MALDI matrix. An increase in the NADH/NAD\(^+\) ratio has been shown to demonstrate a positive correlation with MI\(^{117}\); thus, distinguishing the two forms of this nucleotide may prove beneficial. A MALDI MS spectrum of NAD\(^+\) collected in negative mode is displayed in Figure 3-7. Interestingly, the [M-H]\(^-\) ion of NAD\(^+\) did not appear as the most intense non-matrix ion. Instead, two ions at \( m/z \) 540 and 558, the former ion being more intense, represented the most intense analyte ions. After analyzing the structure of NAD\(^+\), it was concluded that the ion at \( m/z \) 540 likely resulted from the loss of nicotinamide. The ion at \( m/z \) 558 could result from contamination of ADP-Ribose during synthesis of NAD\(^+\).

To confirm the identity of the proposed ion (i.e., \( m/z \) 540), \( \text{MS}^2 \) was performed (Figure 3-8). Following CID of \( m/z \) 540, abundant fragments were observed at \( m/z \) 426,
408, 346, and 328, likely corresponding to the [M-H] of ADP, the [M-H-H$_2$O]$^-$ of ADP, the [M-H]$^-$ of AMP, and the [M-H-H$_2$O]$^-$ of AMP, respectively. Furthermore, the ion at $m/z$ 273 results from a NL of 267, the same as the nominal mass of adenosine. These five fragments present strong evidence for the presence of two phosphate groups bound in series to an adenosine nucleotide. Finally, the ion at $m/z$ 480 (NL of 60) is a common cross-ring cleavage that occurs in the presence of sugars. Thus, taking into account the remaining mass, a ribose ring must also be bound to the two phosphate groups, substantiating the structure proposed in Figure 3-8.

**Comparison of pure 9-AA free base and 9-AA extract**

The supernatant of the extraction detailed above was tested for feasibility as a MALDI matrix. In doing so, the supernatant was either spotted on a MALDI target plate, or mixed with an LPC standard and spotted using the dried-droplet method detailed above. The resulting spots were then analyzed in both positive and negative mode by MALDI MS. A comparison of the pure MALDI matrix and the extract in positive mode is shown in Figure 3-9. A similar comparison for negative mode is shown in Figure 3-10. The spectra from the extract and commercially purchased free base are largely comparable. The sole striking difference appears in negative mode, wherein the ratio of the [M-H]$^-$ ($m/z$ 193) to the [M]+ ($m/z$ 194) appears lower in the extract, though the reason for this phenomenon is currently unclear. The initial HCl salt, dissolved in 70:30 EtOH:H$_2$O (v/v) was also tested, producing similar $m/z$ values to that of the extract and pure free base. Further experiments were conducted analyzing the LPC standard. In both positive and negative ionization modes, MALDI MS analysis utilizing the extract as a matrix provided the expected [M+H]$^+$ or [M-CH$_3$]$^-$ ion for this standard with similar abundance to the commercially available freebase.
Positive mode MS imaging of a rat coronary artery ligation model of myocardial infarction

To evaluate the utility of 9-AA as a positive mode matrix for MALDI MSI, imaging experiments were conducted on transverse sections of cardiac tissue from the rat model for MI. For comparison, serial sections were coated with either 9-AA or DHB. The serial sections were then analyzed sequentially to eliminate any day-to-day variation.

Detected analytes in positive mode

Although 9-AA is primarily utilized for negative mode analyses, a number of analytes can be ionized in positive ion mode with 9-AA as a matrix. In particular, analytes containing a preformed charge (e.g., choline-containing analytes) are still efficiently ionized. Figure 3-11 displays the averaged mass spectra from cardiac tissue coated with either 9-AA (Figure 3-11A) or DHB (Figure 3-11B). A number of common ions (e.g., m/z 496, 524, 782, etc…) are observed in the tissues coated with either matrix. All of the common ions were identified as either PCs, sphingomyelins (SMs), or acylcarnitines. It should be noted that each of these compound classes contains a choline functional group that retains a preformed positive charge on the quaternary amine. The relative abundances of these ions, however, do not appear conserved between 9-AA and DHB-coated cardiac tissue. For example, the three ions at m/z 810, 832, and 848 are thought to correspond to the [M+H]+, [M+Na]+, and [M+K]+, respectively, of PC (18:0/20:4). Analogous to the analysis of the LPC standard, the formation of the [M+H]+ from tissue appears favorable when 9-AA is used, whereas the formation of the [M+Na]+ appears favorable when DHB is used. As alkali adducts were not observed in the LPC standard analysis with 9-AA, the observed adduction is thought to result from the naturally occurring sodium and potassium in the tissue.
Although 9-AA effectively ionized compounds with a preformed positive charge, a number of analytes are absent from the 9-AA spectrum that were previously detected with DHB. For example, positive ions from nucleotides such as adenosine monophosphate and adenosine diphosphate, occurring at m/z 348 and 428, respectively, are noticeably absent from the 9-AA coated tissue. Furthermore, the positive-mode peaks for cholesterol at m/z 369 and heme B at m/z 616 are also absent from the 9-AA spectrum.

Finally, a greater degree of source fragmentation is observed for positive ions with 9-AA than DHB. This phenomenon can be observed via m/z 666 in Figure 3-11A and m/z 725 in Figure 3-11B. Presumably, the ion at m/z 725 corresponds to the [M+Na]^+ of SM (d18:1/16:0) and m/z 666 corresponds to the [M+Na-C3H9N]^+ of the same SM. Although many factors contribute to source fragmentation, laser fluence, source pressure, extraction voltage (restricted to MALDI-ToF MS), and the choice of the MALDI matrix are among the most cited.\textsuperscript{118, 119} As the laser fluence and source pressure were maintained between analyses, the absorptivity of the matrix at 337 nm was thought responsible. The UV-Vis absorption spectra for the DHB and 9-AA matrix solutions are shown in Figure 3-12 and Figure 3-13, respectively. Unlike DHB, a relative minimum exists at 337 nm for 9-AA, suggesting that the weak absorption at this wavelength contributes to the source fragmentation.

**Principal component analysis of perfused and infarcted myocardium**

The utility of 9-AA as a positive mode MALDI matrix for identifying tissue-specific markers of MI was explored. Transverse cardiac sections, coated with either 9-AA or DHB, from a rat LAD ligation model of MI were analyzed in positive ionization mode, and an established multivariate data analysis methodology was applied to determine
markers for the infarcted (damaged) and perfused (healthy) myocardium. In previous studies conducted in this lab, a number of LPCs and PCs were identified as markers for infarcted and perfused myocardium, respectively. Phospholipase A$_2$ (PLA$_2$), an enzyme that hydrolyzes the sn-2 acyl bonds of PCs, was thought responsible for these markers.

Separation of infarcted and perfused myocardium was observed along the first principal component for both 9-AA and DHB-coated tissue (Figure 3-14). The first principal component accounted for a larger percentage of the variance in the 9-AA tissue relative to the DHB tissue (95.8% vs 85.8%, respectively). This difference can be explained by the analytes detected with each matrix. For instance, PCs comprise the vast majority of the positive-mode ion current with 9-AA, whereas PCs comprise a smaller percentage of the ion current with DHB. As most intact PCs contain an unsaturated fatty acid tail in the sn-2 position of the glycerol backbone, and therefore can be acted upon by PLA$_2$, it is not surprising that the spectral variation between the two regions of tissue is greater from the tissue coated with 9-AA relative to DHB.

A one-dimensional loadings plot for the first principal component is displayed in Figure 3-15 for both 9-AA and DHB-coated tissue. Based on a quick inspection, the two loadings plots demonstrate some similar trends; the intact PCs ($m/z$ 750–900) load with the perfused myocardium and the LPCs ($m/z$ 450–600) load with the infarcted myocardium. Furthermore, SM (d18:1/16:0) at $m/z$ 725 and its source fragment at $m/z$ 666 load with the infarcted myocardium following analysis of both tissues. The images for a number of these lipids and the TTC-stained tissue indicating the different tissue regions are displayed in Figure 3-16. For all of these ions, 9-AA produced images of a
quality rivaling DHB. Despite these similarities, a number of small metabolites found to be significant with DHB do not load with either tissue region when 9-AA is utilized. As previously mentioned, the basic nature of the matrix is thought to prevent efficient ionization of these compounds.

**Dicarboxylcarnitines as potential blood-borne biomarkers of myocardial infarction**

Although there are a number of metabolites not detected with 9-AA, the region between \( m/z \) 200 and 450 has an appreciably lower matrix background than DHB. After a closer inspection of the lower mass region for 9-AA, a number of low molecular weight ions (e.g., \( m/z \) 204, 222, 248, and 262) were found to load with the perfused tissue. A comparison of the images obtained for the four labeled compounds in the lower mass region of the 9-AA loadings plot is shown in Figure 3-17. For each of these ions, the tissue coated with 9-AA provided better image contrast between the infarcted and perfused myocardium; two ions \( (m/z \) 222 and 262) did not display any noticeable image contrast between these tissue regions within the DHB-coated tissue. Each of these ions is hypothesized to leak from infarcted myocardium following rupture of the plasma membrane, as previously reported.48

To identify these ions, MS\(^2\) was performed. The lowest molecular weight ion, \( m/z \) 204, demonstrated the highest relative abundance of the four ions. Prior to fragmentation studies, the ion was putatively identified to be the \([\text{M+H}]^+\) ion of acetyl carnitine (C2 carnitine). MS\(^2\) of this ion (Figure 3-18) produced abundant fragment ions at \( m/z \) 60, 85, and 145. The second fragment \( (m/z \) 204→85) is characteristic of all protonated carnitine ions, resulting from successive loss of the fatty acyl chain (in this case acetic acid) and trimethylamine. The ion at \( m/z \) 145, resulting
from a neutral loss (NL) of 59, corresponds to loss of trimethylamine. Furthermore, the ion at $m/z$ 60 corresponds to protonated trimethylamine. Finally, a low abundance fragment ion is observed at $m/z$ 144 (NL 60), resulting from the loss of acetic acid.

A similar fragmentation pattern was observed for $m/z$ 248; however, structures with standard fatty acyl chains could not be assigned—C4 carnitine would exhibit an $[\text{M+H}]^+$ ion at $m/z$ 232 and C6 carnitine would exhibit an $[\text{M+H}]^+$ ion at $m/z$ 260. Instead, the mass-to-charge ratio of this ion suggests an acyl chain amounting to 104 Da. Based on this mass, malonic acid, a dicarboxylic acid, was hypothesized to be esterified to carnitine. The MS$^2$ spectrum in Figure 3-19 supports this hypothesis. Although the most abundant fragments (i.e., $m/z$ 85 and 189) yield little information concerning the fatty acid tail, fragment ions also appear at $m/z$ 87 and 105, corresponding to the $[\text{M+H}]^+$ of dehydrated and intact malonic acid, respectively. In addition, the fragment ion at $m/z$ 144 results from the loss of malonic acid. Similarly, the ion at $m/z$ 262 was hypothesized to contain a fatty acyl chain with a mass of 118 Da. Two possibilities exist for this mass: succinic acid and methylmalonic acid, both of which are present endogenously. The MS$^2$ spectrum for this ion is displayed in Figure 3-20. To date, the exact fatty acyl chain has not been identified; however, for illustrative purposes, the ion is drawn with methylmalonic acid esterified to carnitine. Once again, fragment ions corresponding to the $[\text{M+H}]^+$ of the dehydrated and the intact fatty acyl chain are observed. Although the exact fatty acid structure for $m/z$ 262 has not yet been identified, both C3 and C4 dicarboxylacylcarnitines have been detected from the rat heart.\textsuperscript{120}
**Negative mode MS imaging of a rat coronary artery ligation model for myocardial infarction**

Following positive mode analysis, negative mode MALDI MSI was performed on tissue sections from the rat LAD ligation model for MI. A comparison of the upper half of the heart, stained with TTC, and the MALDI MSI total ion current (TIC) is shown in Figure 3-21. TTC exhibits a color change (from colorless to red) in the presence of mitochondria from healthy tissue. This color change stems from enzymatic reduction of the TTC by dehydrogenases to produce a red formazan. The histological staining in Figure 3-21A demonstrates a fairly large area of infarction (areas of white tissue) near the left ventricle.

Based on the asymmetry of the left ventricle, representative mass spectra were averaged from the infarcted myocardium and the perfused (healthy) myocardium. The averaged mass spectra from these two regions are displayed in Figure 3-22. A number of differences between the two tissue regions can be ascertained by direct comparison of these two spectra. For instance, m/z 540, putatively identified as NAD⁺, appears to show a relatively high intensity in perfused myocardium; however, the same ion does not appear above the background in the infarcted myocardium. In contrast, m/z 1207 demonstrates a higher intensity in infarcted myocardium than perfused myocardium. The images of these two ions are illustrated in Figure 3-23.

**Principal component analysis**

To further analyze these two regions of tissue, the aforementioned multivariate data analysis methodology was applied to spectra extracted from the perfused and infarcted myocardium. Separation between the perfused and infarcted samples was observed along principal component 1, which accounted for 85.3% of the total variance.
Principal component 2, which accounted for 5.5% of the total variance, dictated the variation within a tissue region.

A 1-dimensional loadings plot for principal component 1 was generated to determine ions positively correlating with either of the two regions (Figure 3-25). Ions that load positively on principal component 1 demonstrate a positive correlation with perfused myocardium. Conversely, ions that load negatively on principal component 1 demonstrate a positive correlation with infarcted myocardium. Images for a number of select ions are shown in Figure 3-26. Analysis of the NADH standard suggests that $m/z$ 664, which localizes in the perfused myocardium, is the [M-H]$^-$ of NADH; however, MS$^2$ analysis is necessary to confirm this identification, as is discussed later in the results.

Once markers for the perfused and infarcted myocardium were selected, spectra were also extracted from regions suspected of being at-risk myocardium. These samples were then included in PCA analysis to determine endogenous markers of this tissue region. The scores plot generated from the inclusion of all three tissue regions (infarcted, perfused, and at-risk myocardium) is depicted in Figure 3-27. Separation is observed from all three tissue regions, with principal component 1 (66.6% of the variance) dictating separation between infarcted myocardium and the remaining two tissue regions, and principal component 2 (19.8% of the variance) dictating separation between perfused and at-risk myocardium.

The loadings plot from principal component 1 (Figure 3-28) appears largely similar whether the at-risk myocardium samples are included or not, the major difference being that $m/z$ 1469 loads with the infarcted myocardium when the at-risk myocardium is excluded (Figure 3-25) and conversely loads with the perfused/at-risk myocardium.
when the at-risk myocardium is included. Principal component 2, however, demonstrates a number of features that demonstrate high correlation with the at-risk myocardium. These features, loaded on the negative portion of principal component 2 (Figure 3-29), include a number of low mass ions (e.g., \(m/z\) 244, 403, 565, and 606) in addition to \(m/z\) 1469. The MS images for a few of these ions is displayed in Figure 3-30.

**MS\(^n\) identification and imaging**

Ions that demonstrated both a significant loadings coefficient in PCA analysis and localization in the region of interest were subjected to MS\(^n\) for identification. Initially, targeted experiments within the region of interest were performed. Following identification of the ion, MS\(^n\) imaging experiments were performed, and the characteristic fragment ion of the identified compound was mapped over the entire tissue for localization confirmation. In general, identified compounds were found to belong to one of two compound classes: 1) phospholipids or 2) nucleotides/nucleotide sugars. Both of these classes will be discussed below.

**Phospholipids**

As previously discussed in this dissertation, positive mode analysis of a ligation model demonstrated that phospholipase A\(_2\) (PLA\(_2\)) cleaved the \(sn\)-2 fatty acid tail of intact phospholipids to generate lysophospholipids. Although PLA\(_2\) is thought to have a greater activity towards intact PCs and PE\(_s\), other lipid classes that preferentially ionize in negative mode (e.g., phosphatidylinositolos and cardiolipins) are susceptible to this enzyme as well.

Analysis of the loadings plot in Figure 3-28 yields three ions at \(m/z\) 1447, 1469, and 1485. The 22 amu difference between \(m/z\) 1447 and 1469 suggests that the two
ions differ by replacement of a hydrogen with a sodium atom. Similarly, the 38 amu difference between m/z 1447 and 1485 suggest that the two ions differ by replacement of a hydrogen with a potassium atom. MS² analysis identified these ions as the [M-H]⁻, the [M+Na-2H]⁻, and the [M+K-2H]⁻ of tetralinoleoyl cardiolipin (TLCL). The MS² spectrum for m/z 1447 is shown in Figure 3-31A. The ion at m/z 695 (NL 752) corresponds to phosphatidic acid (18:2/18:2), and the ion at m/z 831 (NL 616) results from the loss of diacylglycerol (18:2/18:2). Furthermore, a low abundance ion at m/z 1167 (NL 262) is observed, corresponding to the loss of dehydrated linoleic acid. The sodium adduct at m/z 1469 demonstrates similar, though not identical fragmentation to the protonated ion (Figure 3-31B). In this instance, the most abundant fragment corresponds to the loss of the diacylglycerol (m/z 853), instead of the ion corresponding phosphatidic acid. Furthermore, three low abundant ions at m/z 1207, 1189, and 1167, corresponding to the losses of dehydrated linoleic acid, linoleic acid, and sodiated linoleic acid, respectively, are observed. Similarly, the potassium adduct of TLCL also demonstrates an abundant loss of the diacylglycerol (m/z 869), and three lower abundant ions at m/z 1223, 1205, and 1167 corresponding to the loss of dehydrated linoleic acid, linoleic acid, and potassiated linoleic acid (Figure 3-31C). The apparent differences in the fragmentation pathways for these three ions (mainly the absence of m/z 695 and 751) are not unexpected, as protonated phospholipid ions and alkyl adducts of phospholipids have been shown to generate vastly different fragment ions under low-energy CID conditions.¹²¹ Analogous to PC ions, fragmentation of the carbon-oxygen bond in the bridging glycerol backbone appears to be inhibited due to the stabilization of either phosphate group by the metal cation.¹²² For further
clarification, the fragmentation pattern for all three of these ions is displayed in Figure 3-32.

As previously noted in Chapter 2, sodium and potassium adducts of phospholipids demonstrate different localization following MI. This phenomenon also occurs with the intact cardiolipins; the protonated ion and potassium adduct localize in the perfused myocardium, and the sodium adduct localizes in both the infarcted and at-risk myocardium. As neither sodium nor potassium was supplemented in the matrix solution or the tissue, these localizations are thought to reflect the natural sodium and potassium content within myocardium. To analyze the overall intact cardiolipin content within the tissue, a summed image of the three cardiolipin ions was generated (Figure 3-33). As can be seen in this image, the overall cardiolipin content appears to decrease despite the apparent increase in sodium content in infarcted myocardium. Similar to intact PCs and PEs, PLA₂ is thought to be responsible for this decreased signal of intact cardiolipins in infarcted myocardium.

Further verification of the enzymatic action of PLA₂ on intact cardiolipins was found with the presence of both mono- and dylsocardiolipins. Unlike most phospholipids, cardiolipins contain two glycerol backbones and four fatty acid tails; thus, there are two sn-2 fatty acid tails available for hydrolysis. To this point, three ions were identified from the loadings plots related to mono- and dylsocardiolipins, all of which positively correlated with the infarcted myocardium. Two of these ions, m/z 1185 and 1207, were identified as the [M-H]⁻ and [M+Na-2H]⁻, respectively, of monolysocardiolipin. Interestingly, the [M+K-2H]⁻ did not demonstrate a significant loadings coefficient with either infarcted or perfused myocardium. The third ion at m/z
945 was identified as the [M+Na-2H]\(^-\) of dilsyocardiolipin, and also demonstrated a positive correlation with infarcted myocardium.

Targeted and imaging tandem MS experiments were conducted to verify the identity and localization of the aforementioned lysocardiolipins. The MS\(^2\) spectrum of dilsyocardiolipin at \(m/z\) 945 is displayed in Figure 3-34. Generally, the lysocardiolipins demonstrated analogous fragmentation to the intact cardiolipins, producing abundant fragment ions resulting from cleavage of P–O bonds, exemplified by the ions at \(m/z\) 591, 507 and 415. Additionally, fragment ions corresponding to either the NL (\(m/z\) 665) of or carboxylate anion (\(m/z\) 279) of linoleic acid were also observed, though less abundant than cleavages about the phosphate groups. MS\(^2\) images mapping the two most abundant transitions for dilsyocardiolipin are also shown in Figure 3-34. Both fragment ions demonstrate localization within the infarcted myocardium.

Although the [M-H]\(^-\) of dilsyocardiolipin did not appear above the baseline in the principal component 1 loadings plot, targeted MS\(^2\) imaging experiments were conducted on \(m/z\) 923 to decouple the effect of salt adduction and localization. That is, if PLA\(_2\) is truly acting upon intact cardiolipins, the [M-H]\(^-\) ion of dilsyocardiolipin should also localize within the infarcted myocardium. To test this hypothesis, an MS\(^2\) imaging experiment was conducted on \(m/z\) 923. The most abundant fragment occurred at \(m/z\) 433, resulting from cleavage at the sn-1 position of the bridging glycerol backbone, (Figure 3-35B. The MS\(^2\) image for this transition (Figure 3-35A) demonstrates a distinct localization with the infarcted myocardium despite the relatively low signal generated. This localization lends credence to the idea that PLA\(_2\) is acting upon intact cardiolipins to form lysocardiolipins.
Although PLA$_2$ has also been reported to act upon phosphatidylinositols (PIs), the most abundant intact PI, PI (18:0/20:4) at m/z 885, did not demonstrate localization in the perfused myocardium. In fact, m/z 885 demonstrated a relatively large loadings coefficient with the intact myocardium. Interestingly, the LPI that results from hydrolysis by PLA$_2$ (m/z 599) also positively correlated with the infarcted myocardium. It is not currently clear as to why the intact PI demonstrates higher signal in infarcted myocardium; however, the generation of the LPI in infarcted myocardium suggests that PLA$_2$ is enzymatically cleaving fatty acids from intact PIs. MS$^2$ experiments fragmenting m/z 599 were conducted to confirm this putative identification. The MS$^2$ spectrum of this ion, displayed in Figure 3-36, demonstrates four abundant fragment ions at m/z 419, 315, 283, and 241. The fragment ion at m/z 419 arises from an NL of 180, presumably due to the loss of inositol, indicative of the PI headgroup. Furthermore, fragment ions are observed at m/z 283 and 315, corresponding to cleavage at the sn-1 position of the glycerol backbone; the fragment ion at m/z 283 is the carboxylate ion for stearic acid and the ion at m/z 315 corresponds to stearic acid as an NL, confirming this ion as the [M-H]$^-$ of LPI 18:0. The most abundant fragment of this precursor ion (i.e., the carboxylate anion at m/z 283) demonstrates localization in the infarcted myocardium, as was indicated by the loadings plot from principal component 1.

Referring back to Figure 3-25 and Figure 3-28, two low abundant masses in the lysoPL region, m/z 480 and 508, consistently load with the infarcted myocardium. These masses are consistent with 3 different lysoPLs, LPC 16:0, LPC 18:0, and LPE 18:0, that were previously identified in positive mode as being generated through enzymatic action of PLA$_2$. Due to the [M-CH$_3$]$^-$ ion formed in negative mode for PCs,
MS analysis is insufficient to determine whether \( m/z \) 480 is LPC 16:0 or LPE 18:0. Accordingly, targeted and imaging MS\(^2\) experiments were conducted within the infarcted myocardium. The MS\(^2\) spectrum for \( m/z \) 480 is displayed in Figure 3-37. Not surprisingly, fragment ions characteristic of both isobaric species are observed; the carboxylate anions for LPC 16:0 and LPE 18:0 are observed at \( m/z \) 255 and 283, respectively. The MS\(^2\) images for these two fragment ions demonstrate localization for the characteristic LPC 16:0 fragment (\( m/z \) 255) within infarcted myocardium. In contrast, the LPE 18:0 fragment (\( m/z \) 283) only displays a slight increase in abundance within the infarcted myocardium. MS\(^2\) analysis of \( m/z \) 508 (Figure 3-38), produced an abundant fragment ion at \( m/z \) 283 and a less abundant fragment at \( m/z \) 224, confirming the ion as the \([\text{M-CH}_3\text{-}]\) of LPC 18:0. Similar to LPC 16:0, the MS\(^2\) image of \( m/z \) 508→283 confirmed that LPC 18:0 was localized within the infarcted myocardium.

**Nucleotides and nucleotide sugars**

A number of \( m/z \) values identified by PCA to have relevant localization within the regions interest were identified by MS\(^2\) as nucleotides. In nearly all instances, the nucleotides were found to contain at least one phosphate group (i.e., nucleoside mono-, di-, or triphosphates) that likely aided in the negative-mode ionization. The most abundant detected nucleotides and nucleotide sugars belonged to the family of adenosine phosphates. The nucleotide phosphates (without an additional bound sugar residue) were observed in negative-mode in the form of \([\text{M-H}]^-\) ions at \( m/z \) 346, 426, and 506 for adenosine monophosphate (AMP), adenosine diphosphate (ADP), and adenosine triphosphate (ATP), respectively. The MS\(^2\) spectrum for ADP at \( m/z \) 426 is displayed in Figure 3-39. For both ATP and ADP, NLs of 98 at 408 and 328, respectively, were observed, suggesting the loss of phosphoric acid in both instances.
Furthermore, in the case of ADP, a fragment ion was observed at $m/z$ 134, suggesting the presence of deprotonated adenine. AMP did not readily lose phosphoric acid, but instead exhibited a NL of 135, corresponding to the loss of adenine to form the product ion at $m/z$ 211. The fragmentation for these three ions indicates that the charge is localized on the phosphate group nearest to adenosine. MS imaging and PCA of all three of these ions demonstrated a marked decrease in signal in the infarcted myocardium, suggesting that these nucleotides are leaching into the bloodstream following myocardial ischemia.

Also within the adenosine family, NADH and NAD$^+$ were putatively identified based on each nucleotide’s nominal $m/z$. Furthermore, both of these ions, $m/z$ 664 and 540 for NADH and NAD$^+$, respectively, exhibited large loadings coefficients, indicating localization in the perfused myocardium. MS$^2$ analysis of the precursor ion at $m/z$ 540, yielded four abundant fragment ions at $m/z$ 426, 408, 346, and 328, confirming this ion as the same species present in the NAD$^+$ standard. Similarly, analysis of $m/z$ 664 produced to abundant fragment ions at $m/z$ 397 and 408, confirming this ion as the [M-H]$^-$ of NADH.

In addition to adenosine-containing nucleotides, several uridine-containing nucleotide and nucleotide sugars, almost exclusively in the form of diphosphates, were detected with significant abundance. The [M-H]$^-$ of uridine diphosphate (UDP) was identified at $m/z$ 403 (Figure 3-40). MS$^2$ of this ion yielded an ion resulting from a NL of 98 at $m/z$ 305. MS$^3$ was conducted on the $m/z$ 403→305 to confirm that this fragment ion is related to UDP. This experiment yielded a single abundant fragment at $m/z$ 111 (NL 194), corresponding to the [M-H]$^-$ of uracil, further validating the assignment of $m/z$
403 as the [M-H] of UDP. Additionally, a fragment ion was observed at \( m/z \) 267 (NL of 136) that is presumably unrelated to UDP. An MS\(^2\) imaging experiment validated this hypothesis, as the two fragments demonstrate different localizations; the fragment ion occurring at \( m/z \) 267 demonstrates localization largely in the perfused myocardium, whereas, the fragment ion occurring at \( m/z \) 305 demonstrated progressively decreasing signal in the at-risk, perfused, and infarcted myocardium.

The increased abundance of UDP in the at-risk myocardium was substantiated by the identification of two UDP-sugars with similar localization. These two sugars, detected at \( m/z \) 565 and 606, were identified as UDP-glucose (UDP-Glc) and UDP-N-acetylglucosamine (UDP-GlcNAc), respectively. The MS\(^2\) spectra of UDP-Glc and UDP-GlcNAc are displayed in Figure 3-41 and Figure 3-42, respectively. CID of both ions yields a fragment ion at \( m/z \) 323 resulting from cleavage between the two phosphate groups. Interestingly, UDP-GlcNAc demonstrates much more extensive fragmentation than UDP-Glc. In addition to the fragment ion at \( m/z \) 323, UDP-GlcNAc yields abundant ions at \( m/z \) 403, 385, 362, and 282, with the fragment ion at \( m/z \) 403 likely resulting from the loss of dehydrated N-acetylglucosamine.

**Conclusions**

This work has demonstrated the utility of 9-AA as a dual-mode matrix for MALDI MSI. 9-AA exhibited more efficient ionization in negative mode relative to DHB. Furthermore, a number of analytes were still detected in positive mode with a signal-to-noise ratio rivaling that of DHB. Perhaps one of the more understated advantages of 9-AA is that the observed crystal size produced is orders of magnitude smaller than DHB, despite the use of a less volatile solvent system (70:30 EtOH:H\(_2\)O vs. 70:30 MeOH:H\(_2\)O.
for 9-AA and DHB, respectively). This crystal size may prove beneficial as MALDI MSI experiments are continually moving towards higher spatial resolution.

Although 9-AA has proved a valuable matrix for both negative and positive mode MALDI MS analyses, the price of the pure free base may detract users from utilizing 9-AA for tissue analysis. This work has demonstrated that a relatively simple and fast acid/base extraction can be used to obtain the free base from the relatively inexpensive hydrochloride hydrate salt of 9-AA. It should be noted that this extraction technique has not yet been optimized; however, any reasonable extraction yields will drive the cost per analysis to rival standard MALDI matrices. Furthermore, it appears that the hydrochloride salt, prepared in the same solvent system as described in this work, may also provide a suitable MALDI matrix for negative mode analyses. This alternative is the optimal solution, as there is no requirement for a liquid/liquid extraction and the price per analysis is approximately 50x lower than using the commercially available freebase. The one caveat identified to this point is the artificial addition of chloride ions to the sample that may ultimately confound analysis of endogenous chloride-containing analytes.

Finally, the analysis of the rat coronary artery ligation model with 9-AA has been extremely valuable in regards to advancing the body of knowledge surrounding lipid chemistry in MI. Firstly, the enzyme PLA$_2$ was found to act upon both intact cardiolipins, intact PIs, and intact PCs in tissue, yielding lysoPLs in areas of myocardial ischemia. To the authors’ knowledge, this is the first instance in which PLA$_2$ has been shown to act upon cardiolipins and intact PIs in situ following MI. Cardiolipins provided a particularly elegant substrate for PLA$_2$, as two $sn$-2 fatty acyl substituents were available
for hydrolysis, evidenced by the formation of dilsocardiolipin in infarcted myocardium. Finally, short-chain dicarboxylcarnitines, detected in positive mode, were also identified as demonstrating a decreased signal in infarcted myocardium. These same analytes were previously overlooked in Chapter 2 using a similar multivariate data analysis methodology with DHB as a MALDI matrix. The relatively low matrix background of 9-AA was instrumental in detecting the changes in these low abundance, low m/z analytes. Interestingly, increased blood-borne concentrations of dicarboxylcarnitines were just recently identified as predictors for future MI events. Thus, we may have only brushed the surface of the role that these low abundant analytes play in MI.

Additionally, a number of nucleotides and nucleotide sugar were found to show dramatically decreased signal in infarcted myocardium. As with the adenosine-containing nucleotides detected in positive mode (Chapter 2), these metabolites are thought to leak from tissue following rupture of the plasma membrane. An unexpected finding within this compound class, however, was that uridine-containing nucleotides (e.g., UDP) were found to localize within the at-risk myocardium. Interestingly, UDP and UTP are known as a positive inotropes (i.e., stimulating muscle contraction). Furthermore, UTP, which has shown to be released into the blood stream following myocardial ischemia, also stimulates vasodilation. Based on this study, it would appear that uridine-phosphates are upregulated under ischemic conditions. Following necrotic cell death, increased levels of these nucleotides may be released into the blood-stream, triggering the cardioprotective aspects of UTP and UDP.
The biological implication of these findings is three-fold. First, this methodology offers an alternative for post-mortem identification of MI via a number of tissue-specific markers of tissue death (necrosis). Second, and more importantly, this methodology has identified a host of small molecule markers of MI, the most valuable of which are water-soluble metabolites that potentially diffuse into the bloodstream. Instead of the typical immunoassays that analyze protein biomarkers that decay within days (or even hours) of the onset of MI, a targeted metabolomics approach utilizing the identified small molecule biomarkers may be useful to determine if a living patient has been afflicted with acute MI. As the accuracy of prediction scales with the number of markers, a large number of low molecular weight markers may prove more accurate than current diagnostic tests using protein biomarkers. Finally, this methodology has provided support for the hypothesis that pyrimidine nucleotides are cardioprotective.
Figure 3-1. Potential negative-mode MALDI matrices for MSI of cardiac tissue.
Figure 3-2. MS$^2$ spectrum of $m/z$ 494 from an LPC 17:0 standard collected in negative mode using 9-AA as a MALDI matrix. The structure and fragmentation pattern of the ion are also shown.
Figure 3-3. Negative-mode MALDI MS$^2$ spectrum of m/z 714 from a synthetic PE (16:0/18:2) standard. The structure of the ion and the ion’s most intense fragments are also indicated.
Figure 3-4. Example of MS$^n$ in negative mode for a PS ion. A) MS$^2$ spectrum of $m/z$ 788 and B) MS$^3$ spectrum of $m/z$ 788$\rightarrow$701 from an l-α-phosphatidylserine extract from porcine brain. The structure of the identified ion, PS (18:0/18:1), and the fragmentation pattern is also shown.
Figure 3-5. MALDI MS spectrum of NADH in negative mode using 9-AA as a MALDI matrix. Source fragments resulting from the deprotonated molecular ion of NADH (m/z 664) are denoted with stars.

Figure 3-6. Negative-mode MALDI MS$^2$ spectrum of m/z 664 obtained from an NADH standard. The fragmentation pattern of NADH is also shown.
Figure 3-7. Negative-mode MALDI MS spectrum of NAD+ standard using 9-AA as a MALDI matrix.

Figure 3-8. Negative-mode MALDI MS$^2$ spectrum of $m/z$ 540 obtained from an NAD standard utilizing 9-AA as a MALDI matrix. The proposed structure and fragmentation pattern for the ion at $m/z$ 540 is also shown.
Figure 3-9. MALDI MS spectra collected in positive ionization mode from the 9-AA extract (Top) and the commercially available freebase (Bottom).
Figure 3-10. MALDI MS spectra collected in negative ionization mode from the 9-AA extract (Top) and the commercially available freebase (Bottom).
Figure 3-11. Averaged MS spectra from cardiac tissue following LAD ligation. Tissue was coated with either A) 9-AA or B) DHB.
Figure 3-12. UV-Vis absorption spectrum of 2,5-dihydroxybenzoic acid dissolved in 70:30 MeOH:H₂O (v/v).

Figure 3-13. UV-Vis absorption spectrum of 9-aminoacridine dissolved in 70:30 EtOH:H₂O (v/v).
Figure 3-14. PCA scores plots separating infarcted myocardium (red triangles) and perfused myocardium (green crosses). Tissue was coated with either A) 9-AA or B) DHB. The circles represent the 95% confidence intervals of the sample groupings.
Figure 3-15. PCA loadings plots from analysis of infarcted and perfused myocardium. Tissue was coated with either A) 9-AA or B) DHB. The positive portion of the first principal component (PC 1) corresponds to ions demonstrating localization in the perfused myocardium and the negative portion of PC 1 corresponds to ions demonstrating localization in infarcted myocardium.
Figure 3-16. MS images from infarcted cardiac tissue. Tissue was coated with either A) 9-AA or B) DHB. Furthermore, C displays the upper half of the heart stained with TTC to visualize the different regions of tissue.
Figure 3-17. MS images generated for low molecular weight ions from ligated hearts. Tissue was coated with either A) 9-AA or B) DHB.
Figure 3-18. MS² spectrum of m/z 204 obtained from perfused cardiac tissue coated with 9-AA as a MALDI matrix. The structure and fragmentation of the identified ion, acetylcarnitine, is also shown.
Figure 3-19. MS² spectrum of m/z 248 obtained from perfused cardiac tissue coated with 9-AA as a MALDI matrix. The structure and fragmentation of the identified ion, malonylcarnitine, is also shown.
Figure 3-20. MS² spectrum of m/z 262 obtained from perfused cardiac tissue coated with 9-AA as a MALDI matrix. The structure of methylmalonylcarnitine, one of the possible isomers at m/z 262, is also shown.
Figure 3-21. Images of a heart following coronary artery ligation. A) Upper-half of the heart stained with TTC and B) total ion current obtained from an MS imaging experiment.
Figure 3-22. Negative mode MALDI MS spectra. The spectra were collected from the A) perfused myocardium and B) infarcted myocardium. The red boxes indicate two ions that are present in higher abundance in either tissue region.
Figure 3-23. MS images of m/z 540 and 1207. The images demonstrate complementary localizations in cardiac tissue following LAD ligation.
Figure 3-24. PCA scores plot from negative mode MALDI MS analysis of infarcted (red triangles) and perfused myocardium (green crosses). The circles represent the 95% confidence interval of the sample groupings.
Figure 3-25. 1-dimensional loadings plot from principal component 1 (PC 1). Ions loaded positively on PC 1 demonstrate a positive correlation with perfused myocardium, and ions loaded negatively on PC 1 demonstrate a positive correlation with infarcted myocardium.
Figure 3-26. Negative-mode MS images of various ions. Images consist of ions loading either negatively (Top) or positively (Bottom) on principal component 1, dictating separation between the perfused and infarcted myocardium.
Figure 3-27. PCA scores plot from negative mode MALDI MS analysis of infarcted (red triangles) and perfused myocardium (green crosses). The circles represent the 95% confidence interval of the sample groupings.
Figure 3-28. 1-dimensional PCA loadings plot of principal component 1 (PC 1) generated from analysis of infarcted, at-risk, and perfused myocardium. Ions loaded positively on PC 1 demonstrate a positive correlation with infarcted myocardium.
Figure 3-29. 1-dimensional PCA loadings plot of principal component 2 (PC 2) generated from analysis of infarcted, at-risk, and perfused myocardium. Ions loaded positively on PC 2 demonstrate a positive correlation with perfused myocardium, and ions loaded positively on PC 2 demonstrate a positive correlation with at-risk myocardium.
Figure 3-30. MS images of various ions loading either on principal component 2, which demonstrates positive correlation with the at-risk myocardium.
Figure 3-31. Negative-mode MALDI MS$^2$ spectra of various high mass ions. The three ions, A) $m/z$ 1447, B) $m/z$ 1469, and C) $m/z$ 1485, were collected from myocardium coated with 9-AA following LAD ligation.
Figure 3-32. Fragmentation pattern for TLCL. The pertinent NLs for the [M-H]⁻, [M+Na-2H]⁻, and [M+K-2H]⁻ are displayed.
Figure 3-33. Summed MS image of $m/z$ 1447, 1469, and 1485 normalized to the total ion current.
Figure 3-34. MS² spectrum of m/z 945, identified as the [M+Na-2H]⁺ of dilysocardiolipin. The proposed fragmentation pattern and MS² images of the two most abundant fragment ions are also displayed.
Figure 3-35. MS^2 analysis of dilsyocardiolipin. A) MS^2 image of m/z 923→433, the most abundant transition for the [M-H]^+ ion of dilsyocardiolipin and B) the structure of this ion demonstrating the pertinent cleavage.
Figure 3-36. MALDI MS$^2$ spectrum of $m/z$ 599, identified as the [M-H]$^-$ ion of LPI 18:0. In addition to the MS$^2$ image of the most abundant fragment ion ($m/z$ 599 $\rightarrow$ 283), the proposed structure and fragmentation of the ion are also shown.
Figure 3-37. Negative-mode MALDI MS$^2$ spectrum of $m/z$ 480. The MS$^2$ images of the two most abundant fragments are shown as an inset.
Figure 3-38. Negative-mode MALDI MS$^2$ spectrum of m/z 508, identified as the [M-CH$_3$]$^-$ of LPC 18:0. The MS$^2$ image of the most abundant fragment is also displayed.
Figure 3-39. Negative-mode MALDI MS$^2$ spectrum of m/z 426. The proposed fragmentation pattern of the identified ion, ADP, and the MS$^2$ image of the most intense fragment is also shown.
Figure 3-40. Negative-mode MALDI MS\textsuperscript{n} identification and imaging of UDP from myocardium. A) MS\textsuperscript{2} spectrum of m/z 403, images of two intense fragments, and the structure of the identified ion, UDP. B) MS\textsuperscript{3} spectrum of m/z 403→305.
Figure 3-41. Negative-mode MALDI MS$^2$ spectrum of m/z 565. The proposed fragmentation pattern of the identified ion, UDP-Glc, is also displayed.
Figure 3-42. MALDI MS$^2$ spectrum of $m/z$ 606 detected from myocardium. The proposed fragmentation pattern of the identified ion, UDP-GlcNAc, is also displayed.
CHAPTER 4
MALDI MS METABOLIC PROFILING OF CAENORHABDITIS ELEGANS

Introduction

Matrix-assisted laser desorption/ionization mass spectrometric imaging (MALDI MSI) is an ideal method to reveal the distribution of biomolecules in tissue.\(^1\) Unlike other mass spectrometry-based surface analysis techniques such as secondary ion mass spectrometry (SIMS) or laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS), MALDI MS provides a method for soft ionization of biomolecules (e.g., lipids, peptides, and proteins).\(^3\) Traditional MALDI MSI methodologies have investigated thin, microtomed tissue sections; however, MALDI MSI has the potential for small organism surface analysis without prior sectioning,\(^{127}\) assuming the organism satisfies the size constraints of typical instruments.

*Caenorhabditis elegans* (*C. elegans*) is a free-living nematode found in soil and compost, and is one of the most widely studied organisms in biological and biomedical sciences. Since the initial methodology describing the mapping of genetic mutants by Sydney Brenner over 40 years ago,\(^{128}\) numerous studies have established *C. elegans* as an important model organism. *C. elegans* is a self-fertilizing nematode with 959 somatic cells.\(^{129}\) Its small size (~1 mm in length), short life cycle (3.5 days), and simple body plan all contribute to the ease of laboratory manipulations, which now have enabled a rich area of research into *C. elegans* chemical biology. These studies, depending on the analyte concentration and analytical methodology, require on the order of 10,000–4,000,000 developmentally synchronized worms grown in liquid culture.\(^{130}\) For example, in one recently reported investigation, exudates (i.e., the exometabolome) from approximately 4,000,000 wild-type and mutant nematodes were
collected with subsequent characterization by NMR and LC-MS.\textsuperscript{130} In addition to the exometabolome, a number of investigations have been conducted using wild-type \textit{C. elegans} and readily available gene-knockouts, characterizing the fatty acid content from fractionated nematode extracts using techniques such as GC-FID or GC-MS.\textsuperscript{131-135} Although a number of significant alterations in fatty acid catabolism were reported, other small molecules that are inefficiently extracted from the nematode surface may not be observed using such methods. Thus, a method that efficiently probes the metabolome of individual \textit{C. elegans} genotypes \textit{in situ} may shed light upon this organism’s chemical biology.

In this report, an alternative approach using MALDI MSI for \textit{C. elegans} chemical biology studies that uses small numbers of individual animals, rather than pooled samples, is presented. There are many advantages to this approach, including minimal sample preparation, a minimal number of specimens needed, and the ability to eventually apply this method to parasitic species that cannot be cultured. To demonstrate the potential for this approach, multivariate analysis was utilized to compare MALDI MSI data from several wild-type and mutant strains.

**Experimental**

**Chemicals and reagents**

2,5-dihydroxybenzoic acid (DHB) was purchased from Acros Organics (Geel, Belgium). 9-aminoacridine (9-AA) was purchased from MP Biomedicals (Solon, OH). HPLC-grade methanol (MeOH), HPLC-grade water (H\textsubscript{2}O), and sodium acetate (NaOAc) were purchased from Fisher Scientific (Fair Lawn, NJ). 100% ethanol (EtOH) was purchased from Decon Labs (King of Prussia, PA). DHB was dissolved in 70:30 MeOH:H\textsubscript{2}O (v/v) to a final concentration of 40 mg/mL with a final concentration of 10
mM NaOAc added to serve as a MALDI matrix. Similarly, 9-AA was dissolved in 70:30 EtOH:H₂O (v/v) to a final concentration of 6 mg/mL; however, NaOAc was not added to the 9- AA matrix solution.

**MALDI MS of nematodes**

Synchronized young adult *C. elegans* strains (wild-type N2, mutant *daf-22*, or mutant *fat-6;fat-7*) were washed three times in distilled H₂O and then pipetted onto a microscope slide and allowed to dry. *C. elegans* were coated with the prepared MALDI matrix (either DHB or 9-AA) by pneumatic spraying with a Type A glass Meinhard nebulizer (Golden, CO). Matrix solution was delivered using a flow rate of 3.0 mL/min and nitrogen was used as a nebulization gas at 30 psi. Three passes were conducted over the microscope slide at a height of approximately 10 cm before allowing a drying time of approximately 15 seconds. The process was repeated until 8 mL (320 mg or 48 mg for DHB or 9AA, respectively) of MALDI matrix solution was deposited atop the microscope slide.

All MALDI MS experiments were conducted utilizing a Thermo Scientific LTQ XL linear ion trap mass spectrometer (San Jose, CA) equipped with a Thermo MALDI ionization source. The MALDI ionization source consisted of a Lasertechnik Berlin MNL 106-LD N₂ laser (Berlin, Germany). The 337 nm laser was operated at a repetition rate of 60 Hz and produced a laser spot size of approximately 100 μm in diameter. The source region of the instrument was maintained at a pressure of 75 mTorr. A laser energy of 4.5 μJ and 3 laser shots per laser stop were utilized for an individual scan. Automatic gain control (AGC) was toggled off during analysis in order to maintain a fixed number of laser shots at each position along the nematode.
Data processing and analysis

MALDI MS spectra were collected over the length of each nematode. In doing so, ten random positions were sampled from head to tail to generate one sample MS spectrum. Within each sample grouping (N2, daf-22, and fat6;fat7), approximately 10 nematodes sampled, and each individual nematode was analyzed in triplicate. Furthermore, mass spectra from areas off the nematodes were collected in triplicate during each experiment to determine background ions resulting from DHB or 9-AA. Mass-to-charge (m/z) and intensity lists were exported from Qualbrowser and saved as .CSV files. Major matrix ions, as determined by experiments collected off the nematode surface, were then removed. The remaining mass features were then normalized to the total ion current (TIC), and each mass feature was mean-centered (i.e., row-wise normalization). The processed data were then imported into Metaboanalyst for multivariate analysis utilizing PCA, as previously reported. 2-dimensional scores plots were generated to visualize the separation of the sample groupings. Loadings, which are the mass intensities that underlie the relationships between the mass spectra given by the scores, were then analyzed to determine important mass features.

MALDI MSI and tandem MS

Following multivariate data analysis, MS imaging was conducted over selected nematodes to determine the localization of mass features with high loadings coefficients on the principal component of interest. In doing so, an oversampling raster step size of 25 μm was utilized. Assuming the mass feature was localized on the nematode cuticle, compound identification for ions with high loadings values was conducted using tandem MS with collision-induced dissociation (CID). In these experiments, MS² imaging experiments were conducted over the entire nematode. Similar to MS imaging, MS²
spectra were collected on the cuticle using a raster step size of 25 μm (oversampling). In all MS and tandem MS experiments, a laser energy of 4.5 μJ and 3 laser shots per spot were utilized. Additionally, the collision energy was maintained at 35 (normalized collision energy %). Following data collection, MS² images were generated for intense fragment ions. Only those ions that were found to localize on the nematodes were used for analyte identification.

Results

In this study, MALDI MS was utilized to characterize the surface metabolic profiles of individual whole *C. elegans*. To validate the approach, three different genotypes were compared: wild-type (N2) and mutant strains *daf-22* and *fat6;fat7*. The *daf-22* mutant lacks a gene that encodes an SCPx homolog, resulting in disruption of peroxisomal fatty acid β-oxidation. Thus, *daf-22* mutants may accumulate long-chain fatty acid lipids on the cuticle. Furthermore, *daf-22* mutants have been shown to be deficient in ascarosides, signaling pheromones that have also been implicated in dauer development. The *fat-6;fat-7* mutants lack two Δ9 desaturase isoforms, and thus demonstrate a decreased overall fatty acid content due to increased fatty acid catabolism. Mutations in *fat-6* and *fat-7* result in a deficiency of 20-carbon polyunsaturated fatty acids as well as oleic acid (18:1) and its derivatives.

MALDI MS profiling of nematode cuticles

MALDI MS was utilized to characterize the compounds naturally occurring on both wild-type N2 and mutant cuticles. Care was taken to obtain average mass spectra along the entire length of the nematode to eliminate spectral variation that may occur due to the area of the cuticle sampled. On average, each nematode was approximately 1 mm in length; thus, 10 mass spectra were required to sample the entire length of the
nematode assuming a laser spot diameter of 100 μm. DHB was initially tested as a MALDI matrix for profiling for all three genotypes. Representative MS spectra from the wild-type nematode (N2) and the gene-knockout nematodes (daf-22 and fat6;fat7) are shown in Figure 4-1.

Analysis of the nematode cuticles coated with DHB demonstrated a number of MALDI matrix-related ions (e.g., m/z 154, 231, 273, and 313), the most intense of which are labeled with an asterisk. Although matrix-related ions are generally uninformative, the wild-type N2 exhibited a relatively high abundance of m/z 231, putatively identified as the [M+2K-H]⁺ of DHB, when compared to both the fat6;fat7 and daf-22 mutants. As potassium salt was not added to the matrix solution, this matrix ion may reflect the potassium content of the nematode. Additionally, a number of suspected phosphatidylcholine (PC) lipid ions were observed on the nematode, namely m/z 184, 542, and 580, putatively identified as the [M+H]⁺ of phosphocholine, the [M+H]⁺ of lysophosphatidylcholine (LPC) 20:5, and the [M+K]⁺ of LPC 20:5. In general, the daf-22 demonstrated a relatively high abundance for both the protonated ion and the potassium adduct of LPC 20:5. Furthermore, the fat6;fat7 nematodes exhibited much lower signal for all LPCs identified in the wild-type N2 and daf-22 gene knockout.

As the previous chapter demonstrated, 9-AA can efficiently ionize PCs in positive mode with comparable sensitivity to DHB. Accordingly, 9-AA was applied to the fat6;fat7 and N2 nematode cuticles to interrogate the PC content. As compared to DHB, the use of 9-AA (Figure 4-2) as a MALDI matrix produced strikingly different mass spectra from the nematode cuticle. In particular, 9-AA demonstrated a significantly lower matrix background in positive mode, with only one appreciable matrix ion at m/z
The basicity of 9-AA ($pK_b$ of approximately 4) is thought to be responsible for this low background; however, the basicity also limits the number of compounds that can be efficiently ionized in positive mode; analytes with a preformed positive charge (e.g., choline and carnitine containing analytes) are among the exceptions. A comparison of the mass spectra from the wild-type N2 and fat6;fat7 mutant cuticles is displayed in Figure 4-2A and 4-2B. The wild-type N2 spectrum produced a number of abundant lyso and intact PCs, with the most abundant at $m/z$ 808, putatively identified as the [M+Na]$^+$ of PC (18:1/18:1). In contrast, the fat6;fat7 mutants demonstrated low signal from both the lyso and intact PCs, and demonstrated relatively high ion signal from $m/z$ 258, 280, and 296, putatively identified as the [M+H]$^+$, [M+Na]$^+$, and [M+K]$^+$, respectively, of glycerophosphocholine (GPC).

**Principal component analysis**

PCA revealed separation between the N2 wild-type and fat6;fat7 mutant nematodes along the first principal component, as evidenced by the scores plot in Figure 4-3. The loadings plot shown in Figure 4-4 reveals the mass spectral variation between the two C. elegans genotypes. The fat6;fat7 mutants are deficient in C18 and C20 mono- and polyunsaturated fatty acids, and thus masses associated with such lipids, most notably, PC (18:1/20:5) at $m/z$ 828 and PC (18:1/18:1) at $m/z$ 808, exhibit an appreciable loadings coefficient with the N2 wild-type nematodes. Masses associated with glycerophosphocholine (i.e., $m/z$ 258, 280, and 296), lacking both the sn-1 and sn-2 fatty acid substituents, loaded heavily with the fat6;fat7 mutants. This finding is supported by previous metabolomics studies of mutant aqueous extracts that have reported increased concentration of glycerophosphocholine in fat mutant strains.
PCA was also applied to data collected from the N2 wild-type and daf-22 mutant strains. Although separation was observed between the two genotypes, typically the within-sample grouping variation was dictated by the first principal component, and a principal component that accounted for a relatively low amount of the variance (typically <5%) dictated separation between the sample groupings (Figure 4-5). Despite the relatively low variance, the loadings plot from the first principal component that dictated separation between the sample groupings was analyzed (Figure 4-6). In general, the daf-22 mutants demonstrated an increase in lysophospholipids (e.g., m/z 542); however, it is currently unclear why this phenomenon occurs.

**MALDI MSI and tandem MS**

Following PCA, an MS imaging experiment was conducted to verify the presence of pertinent m/z values (with high loadings coefficients) on the cuticle. Due to the dimensions of the nematodes, approximately 10 MS spectra can be collected across the nematode using a traditional sampling approach (i.e., rastering the sample with a step size equal to the laser spot diameter). To overcome this sampling issue, an oversampling approach similar to that previously reported\textsuperscript{140} was taken, in which approximately 75% of the previously sampled area was interrogated in each additional scan. In doing so, the number of MS spectra acquired over the nematode cuticle was increased by a factor of 16x. In these experiments, the applied laser energy and number of laser shots were sufficiently low as to not ablate the entire interrogated area, permitting multiple experiments (i.e., MS followed by MS\textsuperscript{2} and MS\textsuperscript{3} analyses) over a single nematode.

Ions that demonstrated localization on the cuticle and an appreciable loadings coefficient on the principal component of interest were then submitted to MS\textsuperscript{n} imaging
analysis for compound identification. In general, two classes of ions were identified by MS\textsuperscript{n}: 1) choline-containing phospholipids and metabolites and 2) adenosine-containing nucleotides. Choline-containing lipids and metabolites have well documented fragmentation patterns. The most abundant choline-containing phospholipid, phosphatidylcholine (PC), exhibits fragmentation characteristic of the PC headgroup; protonated PCs exhibit a characteristic fragment ion at \( m/z \) 184, and alkali adducts of PCs exhibit a characteristic neutral loss (NL) of 59.\textsuperscript{48,91} Adenosine-containing metabolites demonstrate an abundant fragment ion at \( m/z \) 136. Furthermore, adenosine phosphates exhibit fragmentation related to the phosphate group, typically observed as NL of 80 or 98.

Representative images from both the wild-type and mutant nematodes coated with either DHB or 9-AA are shown in Figure 4-7. Imaging analysis of the N2 and \textit{daf}-22 cuticles coated with DHB demonstrated higher abundance for \( m/z \) 542 in the \textit{daf}-22 mutant (Figure 4-7A). MS\textsuperscript{2} analysis of \( m/z \) 542, displayed in Figure 4-8, yielded an abundant fragment ion at \( m/z \) 184, suggesting the presence of a protonated LPC with a single fatty acid chain, eicosapentaenoic acid (EPA, 20:5), esterified to the glycerol backbone. EPA is not an abundant fatty acid observed in phospholipids (PLs) from mammalian tissues; however, previous research has identified EPA as the major fatty acid substituent in PCs for \textit{C. elegans}.\textsuperscript{131,141} The potassium adduct of the same LPC was identified by MS\textsuperscript{2} and MS\textsuperscript{3} at \( m/z \) 580 (Figure 4-9). Fragmentation of this ion yielded a neutral loss (NL) of 59, resulting from cleavage of trimethylamine from the phosphocholine headgroup. Subsequent fragmentation of the resulting ion at \( m/z \) 521 yielded ion signal at \( m/z \) 397 (NL of 124), resulting from cleavage of the remainder of
the phosphocholine headgroup. Furthermore, the fragment ion at $m/z$ 163 has been identified as potassiated cyclophosphane, confirming this ion as a potassium adduct. With this knowledge, the remaining mass of the precursor ion can be attributed to the fatty acid substituent, the glycerol backbone, and the hydroxyl group in the $sn$-2 position of the glycerol backbone. Therefore, the ion was identified as the $[M+K]^+$ of LPC 20:5. Other known abundant fatty acids in *C. elegans* (e.g., 18:1 or 17∆) were also found to be present in LPCs using a similar identification strategy.

Imaging analysis of the N2 and *fat6;fat7* cuticles coated with 9-AA demonstrated a higher abundance for both intact and lyso PCs in the wild-type N2 nematodes (Figure 4-7B). Specifically, lyso and intact PCs that contain oleic acid (18:1) in either the $sn$-1 or $sn$-2 position of the glycerol backbone were found to be depleted in the *fat6;fat7* mutants, as evidenced by the images for $m/z$ 808 (putatively identified as the $[M+Na]^+$ of PC (18:1/18:1)). Accordingly, MS$^n$ experiments were conducted to verify the putative assignments of ions that dictated separation between the mutant and wild-type nematodes. Similar to the other alkali adducts of PCs interrogated, $m/z$ 808 demonstrated an abundant NL of 59 following CID. MS$^3$ of $m/z$ 808→749 (Figure 4-10) demonstrated an abundant neutral loss of 124, once again corresponding to the loss of the remainder of the phosphocholine headgroup. Lower in abundance, the NL of 146 suggested the presence of sodium, rather than potassium, acting as the alkali metal adducting with this lipid. Finally, the ion at $m/z$ 467, corresponding to a NL of 282, presumably resulted from cleavage of oleic acid from the $sn$-1 position, and to a lesser extent the $sn$-2 position of the glycerol backbone. A summary of the major ions loading with either the wild-type N2 or *fat6;fat7* double mutants is presented in Table 4-1.
In contrast to the intact and lyso PCs, the fat6;fat7 nematodes demonstrated a higher abundance for free glycerophosphocholine (GPC) on a single worm basis (Figure 4-7B). This metabolite has hydroxyl groups in lieu of fatty acids in both the sn-1 and sn-2 positions of the glycerol backbone. MS$^2$ analysis of m/z 258 (the [M+H]$^+$ of glycerophosphocholine) confirmed this assignment, as a single abundant fragment ion was observed at m/z 104, corresponding to protonated choline (Figure 4-11).

In addition to the choline-containing lipids and metabolites mentioned above, a series of ions at m/z 348, 428, and 508 were observed with relatively high signal on the DHB-coated nematode cuticle (Figure 4-12A and Figure 4-12B). The 80 Da increase in mass with each successive ion indicated an increasing number of phosphate groups bound to a central moiety. After further investigation, these ions were found to be adenosine monophosphate (AMP) in its linear form, adenosine diphosphate (ADP), and adenosine triphosphate (ATP), in order of increasing m/z. All of these ions exhibited a characteristic fragment ion at m/z 136 upon CID that localized on the nematode cuticle (Figure 4-12C and Figure 4-12D). This fragment ion is stably stored in the linear ion trap when fragmenting both AMP and ADP; however, m/z 136 is not stored under normal experimental conditions ($q_{activation}$=0.25 for m/z 508) for the higher mass ATP ion at m/z 508. To observe this fragment ion for ATP, the q of activation for m/z 508 was decreased to 0.23. In addition to the characteristic fragment ion at m/z 136, ADP and ATP exhibited fragmentation relating to phosphate groups, with characteristic NLs of 80 and 98 from both ions.

Conclusions

This work has demonstrated the detection of endogenous compounds directly from the nematode cuticle using positive mode MALDI MSI. In doing so, a number of
low molecular weight PLs and metabolites were identified. These analytes were detected directly from intact nematodes without prior extraction or alteration other than matrix application. The detected PLs are likely resulting from the lipid-rich epicuticle, the outermost layer of C. elegans. The fatty acid configuration in both the lysoPLs and the intact PLs agree well with previous reports detailing the analysis of fatty-acid methyl esters from nematode extracts. Furthermore, distinction between wild-type and gene-knockouts was achieved by utilizing MALDI MSI in conjunction with PCA. The loadings plot of the first principal component, which differentiated wild-type and fat6;fat7 double mutants, substantiates previous reports that there is higher fatty acid content in the wild-type as compared to the fat double mutants. PCA also demonstrated that the fat6;fat7 mutants were deficient in many PC species containing unsaturated fatty acids (e.g., 18:1), and that the mutants also exhibited a higher content of glycerophosphocholine. A similar phenomenon has been observed in metabolomics analysis of aqueous and lipid extracts by both NMR and LC-MS.

Thus, this research has demonstrated the utility of MALDI MS to perform metabolic profiling of whole organisms, such as C. elegans, without the need for prior sectioning. As opposed to previous profiling techniques that utilize on average 4–5 orders of magnitude more biological specimens, this methodology has determined biologically relevant chemical alterations resulting from genetic mutations with as few as 10 biological replicates. In addition, the time required for both sample preparation and analysis was drastically reduced as compared to LC-MS profiling of the exudates. Although this research was performed using commercially available mutants that are
readily cultured, the work presented demonstrates the potential of MALDI MS to
determine metabolic profiles for organisms that are either rare or impossible to culture.
Figure 4-1. Representative MS spectra from *C. elegans* utilizing DHB as a MALDI matrix. Three genotypes are displayed, including A) the wild-type N2, B) the *fat6;fat7* and C) *daf-22* mutant cuticles. Ions known to result from the MALDI matrix are labeled with an asterisk.
Figure 4-2. Representative MS spectra from *C. elegans* utilizing 9-AA as a MALDI matrix. Two genotypes are displayed, including A) the wild-type N2 and B) the *fat6;fat7* mutant cuticles. Ions known to result from the MALDI matrix are labeled with an asterisk.
Figure 4-3. PCA scores plot describing the separation between wild-type N2 (green crosses) and fat6;fat7 double mutant (red triangles) C. elegans.
Figure 4-4. PCA loadings plot from principal component 1 dictating the separation between the N2 (positive) and fat6;fat7 (negative) C. elegans strains.
Figure 4-5. Principal component analysis scores plot (PC1 vs. PC4) detailing the separation between the wild-type N2 and daf-22 gene knockout. DHB was utilized as a MALDI matrix. The ovals represent the 95% confidence interval of the sample groupings.
Figure 4-6. PCA loadings plot from principal component 4 dictating the separation between the N2 (positive) and *daf-22* (negative) *C. elegans* strains.
Figure 4-7. MS images of wild-type (Left) and mutants (Right) for various ions (structures shown) detected from the nematode cuticle. A displays a comparison of N2 and daf-22 using DHB as a MALDI matrix and B displays a comparison of N2 and fat6;fat7 using 9-AA as a MALDI matrix.
Figure 4-8. MALDI MS\(^2\) spectrum obtained from a wild-type N2 nematode. The proposed structure of the ion, LPC 20:5, and the fragmentation pattern is also shown.
Figure 4-9. MALDI MS^n structural elucidation of m/z 580, identified as the [M+K]^+ of LPC 20:5. A) MS^2 spectrum of m/z 580 and B) MS^3 spectrum of m/z 580→521 obtained from a wild-type N2 nematode.
Figure 4-10. MALDI MS$^3$ spectrum of m/z 808→749 collected from a wild-type N2 nematode. The structure of the identified ion, the [M+Na]$^+$ of PC (18:1/18:1), and the fragmentation pattern is also displayed.
Figure 4-11. MALDI MS² spectrum of m/z 258 collected from a mutant fat6;fat7 nematode. The structure and fragmentation of the proposed ion, the [M+H]⁺ of glycerophosphocholine, is also displayed.
Table 4-1. List of compounds that correlate strongly with the wild type N2 (positive loadings coefficient) or fat6;fat7 (negative loadings coefficient) nematodes.

<table>
<thead>
<tr>
<th>m/z</th>
<th>Identification</th>
<th>Ion</th>
<th>Loadings</th>
</tr>
</thead>
<tbody>
<tr>
<td>162</td>
<td>l-carnitine</td>
<td>[M+H]^+</td>
<td>-0.036</td>
</tr>
<tr>
<td>184</td>
<td>Phosphocholine</td>
<td>[M+H]^+</td>
<td>0.313</td>
</tr>
<tr>
<td>258</td>
<td>Glycerophosphocholine</td>
<td>[M+H]^+</td>
<td>-0.651</td>
</tr>
<tr>
<td>280</td>
<td>Glycerophosphocholine</td>
<td>[M+Na]^+</td>
<td>-0.371</td>
</tr>
<tr>
<td>296</td>
<td>Glycerophosphocholine</td>
<td>[M+K]^+</td>
<td>-0.362</td>
</tr>
<tr>
<td>522</td>
<td>LPC (18:1)</td>
<td>[M+H]^+</td>
<td>0.066</td>
</tr>
<tr>
<td>544</td>
<td>LPC (18:1)</td>
<td>[M+Na]^+</td>
<td>0.064</td>
</tr>
<tr>
<td>560</td>
<td>LPC (18:1)</td>
<td>[M+K]^+</td>
<td>0.025</td>
</tr>
<tr>
<td>786</td>
<td>PC (18:1/18:1)</td>
<td>[M+H]^+</td>
<td>0.068</td>
</tr>
<tr>
<td>806</td>
<td>PC (18:1/20:5)</td>
<td>[M+H]^+</td>
<td>0.093</td>
</tr>
<tr>
<td>808</td>
<td>PC (18:1/18:1)</td>
<td>[M+Na]^+</td>
<td>0.153</td>
</tr>
<tr>
<td>824</td>
<td>PC (18:1/18:1)</td>
<td>[M+K]^+</td>
<td>0.065</td>
</tr>
<tr>
<td>828</td>
<td>PC (18:1/20:5)</td>
<td>[M+Na]^+</td>
<td>0.143</td>
</tr>
<tr>
<td>844</td>
<td>PC (18:1/20:5)</td>
<td>[M+K]^+</td>
<td>0.072</td>
</tr>
</tbody>
</table>
Figure 4-12. MS² elucidation of AMP. A) MS image of $m/z$ 348 normalized to the TIC, B) optical image, and C) MS² image of $m/z$ 348→136 of a wild-type N2 nematode. The MS² spectrum of $m/z$ 348 and the structure of the identified ion (AMP) is shown in D.
CHAPTER 5
SUMMARY AND FUTURE WORK

Summary

This dissertation has presented multivariate data analysis strategies for two biological applications of MALDI mass spectrometric imaging datasets. Initially, a guided multivariate data analysis methodology was developed using a model system simulating myocardial infarction. Transverse cardiac sections from this model, also known as a 24-hour coronary artery ligation, proved to be extremely valuable for method development, as a number of spatially segmented and spectrally distinct regions of interest were present. The methodology utilized dimensionality reduction techniques, namely principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA), to efficiently sift through large imaging datasets and extract relative biochemical markers for each region of interest.

Application of this technique to the model system produced results consistent with the preexisting body of knowledge for myocardial infarction. Mainly, the activity of phospholipase A_2 was confirmed in areas of infarcted myocardium. Furthermore, the presence of lipid droplets in the at-risk myocardium was confirmed via the presence of elevated triglyceride concentration. In addition, a number of novel water-soluble metabolites were identified as potential blood-borne biomarkers for myocardial infarction.

Although a number of lipidomic and metabolomic markers for myocardial infarction were identified with the established methodologies, experiments in Chapter 2 of this dissertation were limited to positive ionization mode with the matrix 2,5-dihydroxybenzoic acid. Chapter 3 of this dissertation describes a body of research
proposing the use of 9-aminoacridine (9-AA) as a dual purpose (positive and negative mode) MALDI matrix for the study of lipids and metabolites in the same model system. Although predominantly a negative-mode matrix, 9-AA proved to be an effective MALDI matrix for the ionization of phosphatidylcholines and acylcarnitines in positive ionization mode. A low matrix background, coupled with the relative basicity of the matrix, served to suppress chemical noise often associated with MSI experiments on tissue. By applying the methodologies developed in Chapter 2, dicarboxylacylcarnitines were identified as potential blood-borne biomarkers of myocardial infarction. Furthermore, serial sections, coated with DHB for comparison, exhibited similar phosphatidylcholine localization to that of 9-AA coated sections, thereby validating this matrix for positive mode ionization.

The same methodology was also applied to the ligation model in negative ionization mode using 9-AA as a MALDI matrix. The matrix proved to be effective for the ionization of a broad range of analytes, including anionic lipids (e.g., cardiolipins and phosphatidylinositol), nucleotides (e.g., adenosine monophosphate and NADH), and even select cationic lipids (e.g., lysophosphatidylethanolamines and lysophosphatidylcholines). Multivariate data analysis conducted on the various regions of myocardium confirmed the ability of phospholipase A₂ to act on both cardiolipins and phosphatidylinositol. Furthermore, a number of nucleotides were identified as potential blood-borne markers for myocardial infarction. Although most nucleotides demonstrated localization in the perfused myocardium, the uridine nucleotides appeared with increased abundance in the at-risk myocardium. This localization has never before been reported; however, various clinical trials have identified uridine
nucleotides as potential pharmacological agents to limit tissue loss. Thus, we hypothesize that the presence of uridine nucleotides in the at-risk myocardium may have mechanistic implications for the tissue’s response to myocardial ischemia.

The final research chapter of this dissertation described a methodology for determining the metabolic profiles from Caenorhabditis elegans (C. elegans). In this work, three genotypes, a wild-type and two genetic mutants, were analyzed. Once again, a combination of MALDI MSI and multivariate data analysis was utilized to identify metabolic differences arising from the different genotypes. To increase the number of samples collected from a nematode, an oversampling approach was conducted. Utilizing this approach, biologically relevant biochemical distinctions between the genotypes were identified. Furthermore, these results were obtained from the analysis of individual nematodes, as opposed to thousands, or even millions of nematodes, as previously reported.

**Future Work**

**Time course and reperfusion studies of myocardial infarction**

Due to the limited availability of biological samples, MSI experiments were performed on only one time point (24 h) following administration of the LAD coronary artery ligation surgery. A more detailed study analyzing multiple time points, both before and after the 24 h time point, may yield information concerning the progression of myocardial infarction and the myocardium’s short- and long-term response to the condition. In particular, understanding how myocardium responds to the loss of oxygenated blood over time via at-risk myocardium markers may result in the development of pharmaceuticals to limit myocardial tissue loss. Additionally, studies that map the area of infarction at various time points immediately following ligation
surgery may lead to a greater understanding of optimal pharmaceutical delivery time and the expected tissue loss as a result of delay between onset and administration.

In addition to studying the progression of MI and tissue response to MI, it would be valuable to study the effect of reperfusion (i.e., the restoration of oxygenated blood to previously ischemic areas of myocardium) on the viability and long-term recovery of myocardium. Currently, the initial therapeutic response applied to a patient that has experienced MI is to perform some form of reperfusion, such as thrombolysis, angioplasty, or in severe cases, bypass surgery.\textsuperscript{142} The restoration of blood flow to the previously occluded artery results in a smaller final infarct size, and consequently a lower mortality rate for a given population.\textsuperscript{143} The administration of reperfusion is unfortunately not without risk. The immediate restoration of oxygenated blood to myocardium may result in cell death in myocardium that was reversibly injured prior to reperfusion.\textsuperscript{143} This process is widely known throughout the medical field as “ischemia/reperfusion injury”. Although the beneficial effects of reperfusion appear to outweigh the negative effects, many cardiologists believe that it is possible to minimize ischemia/reperfusion injury via intervention. However, before an optimal intervention strategy can be formulated (e.g., postconditioning or pharmacological intervention), a better understanding of the biological processes occurring following reperfusion is needed. The methodologies developed in this work offer a promising opportunity to study the biochemical mechanism of ischemia/reperfusion injury. Furthermore, these methodologies provide an effective means to evaluate intervention methods for ischemia/reperfusion injury.
Correlation of MSI and LC-MS data

Future work will also focus on correlating MSI and LC-MS results from the ligation model. At present, quantitation by MALDI MSI remains difficult; however, there are many established methods for quantitation of tissue extracts and plasma samples by LC-MS. A comparison of the results from both methods would serve as an effective validation for the methodologies presented in this dissertation. In particular, the quantitative analysis of tissue extracts would yield confirmation on the tissue-specific biological pathways mentioned in this report. Furthermore, the ability to analyze plasma from the same biological specimen, as compared to a control specimen, would offer confirmation for the potential blood-borne markers of MI proposed in this work.

Improving MALDI MSI spatial resolution on the MALDI Thermo LTQ XL via reduction of the laser spot size

The spatial resolution of MALDI MSI experiments is theoretically limited by three factors: 1) the raster step size of the sample stage, 2) the size of the matrix/analyte cocrystals, and 3) the spot diameter of the ionization source. Of the three limitations, the raster step size of the sample proves to be insignificant on modern instruments. Furthermore, recent MALDI matrix coating techniques have produced crystal sizes much smaller than the laser spot diameter of the source utilized in this research. Thus, the laser spot diameter is currently the major hindrance for performing high spatial resolution MALDI MSI experiments.

There are two prevailing strategies for reducing the effective spot diameter of a UV-laser. The first strategy involves using an aperture to allow a small portion of the laser beam to reach the sample surface, and the second strategy involves using optics to reduce the beam diameter. Although the simpler approach, the use of an aperture,
reduces the total energy incident on the surface by a factor of the radius squared; however, the laser fluence (J/cm²) is retained. This decrease in total energy may result in significant losses in sensitivity. In contrast, reducing the spot diameter of the laser optically retains the total energy incident on the surface, but with an increase in laser fluence that may lead to a greater degree of source fragmentation.

Preliminary efforts have attempted to implement a Galilean beam expander\textsuperscript{144} on the Thermo LTQ XL, of which the standard optical configuration is displayed in Figure 5-1. A Galilean beam expander, detailed in Figure 5-2, consists of two lenses: a planoconcave lens and a planoconvex lens. The planoconcave and planoconvex lenses are arranged so that the true focal point of the planoconvex lens and the virtual focal point of the planoconcave lens coincide in space. The result is that the incident beam is continually expanded by the planoconcave lens until contacting the planoconvex lens, wherein the diameter is conserved, following the same angle as the incident beam. The expansion, or magnification ratio, of the configuration is determined by the ratio of the two focal points. The magnified beam can then be focused onto the sample target by a convex focusing lens. Assuming the focusing lens is larger than the incident beam, the incident beam diameter is proportional to the numerical aperture (NA), as the beam diameter directly determines the half angle between the outermost portion of the beam and the focal point (Equation 5-1).

\[ NA = n \sin \theta \]  

(5-1)

Furthermore, the numerical aperture is inversely proportional to lateral spatial resolution (\( \Delta x \)) and directly proportional to the wavelength of irradiation (\( \lambda \)), as detailed in Equation 5-2.
\[ \Delta x = 1.22 \frac{\lambda}{NA} \]  

(5-2)

By substituting Equation 5-1 into Equation 5-2, one can see that by increasing \( \theta \), progressively smaller lateral resolutions can be achieved. Thus, an increase in the beam diameter prior to the focusing lens will produce a concurrent decrease in the spot size on the MALDI target plate.

The most logical implementation of this configuration on the LTQ XL is the optical rail between the UV and dichroic mirrors. The optical rail provides a stable horizontal axis that allows fine adjustment of the interlens distance. Furthermore, the rail forces the two lens faces to be aligned within the plane of the mirrors. An added advantage is that this region of the MALDI source is not held under vacuum, and can easily be accessed for implementation or removal of the lenses.

Although the lenses have been obtained, slight imperfections in the machining of the laser optics module have resulted in an optical rail that is not absolutely level. Furthermore, the current LTQ optical configuration allows few degrees of freedom of alignment; the UV mirror can only be adjusted in the vertical dimension and the dichroic mirror can only be adjusted in the horizontal dimension. Due to the aforementioned flaws, implementation of the beam expander resulted in an enlarged beam that was not centered in the convex focusing lens. The focused beam would then miss the hole in the transfer quadrupole (Q00), and not reach the target plate. We hypothesize that the beam is vertically off-center, as the entire range of the horizontal dichroic mirror was tested. Unfortunately, the LTQ does not permit coarse vertical tuning of the beam following the optical rail, and any skewing of the beam by the UV mirror would result in a
misaligned beam travelling through the beam expander. Thus, an alternative method must be implemented to use this beam expander.

**Outlook**

MSI is an analytical technique that is rapidly gaining utilization for a breadth of applications. Specifically, MSI has inherent utility for characterization of diseases that produce spatially localized biochemical alterations within tissue. Over the course of this research, the MSI community has achieved great strides in not only multivariate data analysis, but also quantitative analysis and high spatial and spectral resolution instrumentation. The ability to combine multivariate data analysis with high resolution MSI will eventually permit the rapid analysis of multidimensional datasets at previously uncharted spatial and spectral resolutions.
Figure 5-1. Standard optical Configuration of the MALDI source interfaced with the Thermo Scientific LTQ XL.
Figure 5-2. Representation of a Galilean beam expander.
Figure 5-3. Schematic of LTQ optical configuration with Galilean beam expander implemented on the optical rail. The spacing between the planoconcave and planoconvex lenses is 10 cm.
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BIOGRAPHICAL SKETCH

Robert Francis Menger was born in 1986 to Elaine and Arthur Menger, the second of two children. He lived in East Islip, NY prior to attending Wake Forest University for college. At Wake Forest, Robert received a Bachelor of Science in chemistry and a Minor in mathematics. While studying capillary electrophoresis under Dr. Christa Colyer, Robert received the Eastern Analytical Symposium award for undergraduate research, and ultimately decided to pursue a career in analytical chemistry research. Following completion of his undergraduate studies, Robert joined the Richard A. Yost research group at the University of Florida to work in mass spectrometric imaging. Under the direction of Dr. Richard A. Yost, Robert received the Roger and Jo Bates fellowship. During his studies, Robert has twice traveled to the FOM Institute for Atomic and Molecular Physics in Amsterdam, the Netherlands, to study cutting-edge mass spectrometric imaging and statistical methods under the supervision of Professor Ron Heeren. Throughout his research career, Robert has developed a passion for studying biological systems on a molecular level, and he looks forward to new and exciting opportunities to pursue this passion following graduate school.