Desorption Electrospray Ionization Mass Spectrometry Imaging: Instrumentation, Optimization and Capabilities

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Desorption Electrospray Ionization Mass Spectrometry Imaging: Instrumentation, Optimization and Capabilities

Manan Dhunna

A thesis submitted to the faculty of Brigham Young University in partial fulfillment of the requirements for the degree of Master of Science

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ABSTRACT

Desorption Electrospray Ionization Mass Spectrometry Imaging: Instrumentation, Optimization and Capabilities

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Master of Science

Desorption Electrospray Ionization Mass spectrometry Imaging (DESI-MSI) is an area of great interest and a promising tool in the field of chemical imaging. It is a powerful, label-free technique, which can determine, map and visualize different molecular compounds on a sample surface. The amount of information acquired in a single DESI-MSI experiment is enormous compared to other techniques, as it can simultaneously detect different compounds with their spatial distribution on the surface. The experiment can be used to produce two-dimensional and three-dimensional images. Chapter 2 focuses on the design and optimization of the setup for performing DESI-MS imaging on various substrates. The proposed setup was tested for its lateral spatial resolution. To provide proof-of-concept of the design, preliminary tests were performed to generate images from commercial thin layer chromatographic plates and photographic paper. Chapter 3 focuses on demonstrating the compatibility of novel microfabricated Thin Layer Chromatography plates (M-TLC plates) for detection with DESI-MSI.

Keywords: Desorption Electrospray Ionization, DESI, Desorption electrospray Mass Spectrometry Imaging, DESI-MSI, Microfabricated Thin Layer Chromatography, M-TLC, Ambient Ionization Mass Spectrometry
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1.1. **Introduction**

Mass spectrometry imaging (MSI) is an area of great interest and a promising tool in the field of mass spectrometry. It is a powerful label-free technique, which can identify, map, and visualize different molecular compounds on a sample surface. The amount of information acquired in a single MSI experiment is enormous compared to other techniques, as it can simultaneously detect different compounds with their spatial distribution on the surface. The experiment can be used to produce two-dimensional and three-dimensional images. The potential of MSI has been explored in fields like marine products, biomedical sciences, and natural products.\(^1\)-\(^4\)

Sample preparation, data acquisition, and data analysis are the three steps in mass spectrometry imaging. Sample preparation greatly depends on the ionization technique used. For example, with Matrix Assisted Laser Desorption Ionization (MALDI)\(^5\) and Secondary Ion Mass Spectrometry (SIMS)\(^6\), a lot of sample preparation is required, whereas in Desorption ElectroSpray Ionization (DESI)\(^7\), which is an ambient ionization technique, sample preparation requirements are negligible. The data acquisition step includes desorption and ionization of analytes, separation of the analytes according to their m/z ratios by a mass analyzer, and detection. Two different ways of performing the desorption and ionization step in MSI are a) microprobe mode and b) microscope mode.

1) **Microprobe** – The microprobe method encompasses a small, localized region of the sample subjected to a focused ion beam, typically of micron or submicron dimensions. This is done
successively for an array of discrete points by scanning the microprobe beam across the sample surface in a raster pattern. A mass spectrum is obtained at each point and stored digitally. Using appropriate software, the images are reconstructed for each mass from the individual mass spectra. The rastering of the ionization beam can be done by moving the stage on which the sample is placed and by keeping the beam steady or by rastering the beam itself and keeping the stage in place. One of the limitations of microprobe method is the loss of the spatial information within the localized ionization spot. Thus the 2-D resolution is limited by focusing of the ionizing beam.\textsuperscript{2, 8} 

2) \textit{Microscope} – The microscope method of imaging is not as commonly used as the microprobe method. The microscope method utilizes ion optics, an analyzer and a position sensitive detector to generate spatial information from the sample. In addition to intensity and the m/z, it also provides information about the original relative positions of the ions on the sample surface. In contrast to microprobe mode, it provides spatial information from the localized spot and is independent of the ionization beam, and thus has the advantage of superior resolution. The performance depends upon the magnification of the microscope, the quality of the ion optics and the resolution of the position sensitive detector.

Data acquisition is followed by data analysis, which is also a multi-step process. First the recorded mass spectra are converted to a 2D image file with the help of software such as MSconvert (freeware, www.ProteoWizard.com) and imzML converter (freeware, www.maldi-msi.org). Then the 2D image file is opened and visualized in either freely available software such as Biomap (freeware, www.maldi-msi.org) or Datacube Explorer (freeware, www.maldi-msi.org), or commercial software like Fleximaging (http://www.bruker.com/) or Firefly (http://www.prosolia.com). In all the visualization software multiple images from the same file
can be opened at the same time based on the selected m/z values. In the 2D or 3D ion images produced, the relative intensities of the ions are indicated by false colors, for which a false color intensity scale bar is provided. Using that same scale the cutoff values for ion intensities to be displayed can also be set. Finally there are features in the software that can perform complex functions on the data set, such as multivariate statistical analysis, baseline correction, etc.

1.2. **Traditional Techniques for Imaging Mass Spectrometry**

The first step to perform imaging mass spectrometry is ionization of the analyte followed by detection in the mass spectrometer. There are various ionization techniques that have been developed for this purpose, including Secondary Ion Mass Spectrometry (SIMS) and Matrix Assisted Laser Desorption Ionization (MALDI). SIMS was the first ionization technique used for imaging purposes, followed by MALDI. Since SIMS and MALDI both can only be operated in vacuum and MALDI needs extensive sample preparation before the desorption/ionization step, they are difficult to work with. This difficulty led to extensive research in the field of ambient mass spectrometry, which requires negligible sample preparation and is easier to use than MALDI and SIMS. Desorption ElectroSpray Ionization (DESI) is the ambient ionization technique that has been most extensively used for MSI. SIMS and MALDI, on the other hand, can have superior resolution, resolving features as small as 100 nm and 10 μm respectively. They are still the technique of choice for high-resolution experiments. The best resolution reported to date with DESI is 35 μm under controlled conditions. There is usually a tradeoff between resolution and analysis time, the two being directly proportional to each other. With an increase in spatial
resolution, there is a corresponding increase in analysis time. Therefore to solve the addressed problem the minimum resolution required for the experiment should be chosen.

1.2.1. SIMS

SIMS was invented by Alfred Benninghoven over 40 years ago and is a widely-used ionization technique for IMS. It is a sensitive surface analysis method in which the analyte is bombarded with a focused beam of ions, which is known as the primary ion beam. The collision of the primary ion beam with the surface results in deposition of energy on the surface, followed by subsequent ejection of mostly neutral particles, along with a small fraction of charged particles, which are known as secondary ions. These secondary ions are collected and analyzed by the mass spectrometer, and elemental and molecular characteristics of the analytes are obtained. The primary ion beam energies used vary from 10 keV to 30 keV, and a number of ions or clusters have been used for collision, including Ar$^+$, Xe$^+$, Cs$^+$, O$_2^+$ and Au$^+$.\textsuperscript{13-15} SIMS has been divided into two classes depending upon the ion beam intensity. A) Static SIMS uses a low intensity primary beam with less than $10^{13}$ ions/cm$^2$, which probes just one or two top layers, and causes minimal sample damage. B) Dynamic SIMS, on the other hand employs beams with greater than $10^{13}$ ions/cm$^2$, which results in sample sputtering. Dynamic SIMS is a destructive technique that allows depth profiling from nanometers to several micrometers.

Applications of SIMS-MSI include the imaging of phospholipids, glycerol and diglycerides in mouse brain tissue and mouse muscular cells using static TOF- SIMS.\textsuperscript{16} Sulfatides and phosphoinositols from mouse brain sections were also studied using TOF-SIMS.\textsuperscript{17} SIMS-MSI has also been used to perform imaging of a number of low mass ions such as Na$^+$, K$^+$ and Ca$^{2+}$ in
cells to study the physiology of diseased cells.\textsuperscript{18,19} The distribution of small molecules in steatotic liver has also been studied using SIMS. Chandra et Al. used SIMS to visualize mitotic spindles from T98G human glioblastoma tumor cells.\textsuperscript{18}
Figure 1.1 Schematic representation of SIMS.
1.2.2. MALDI

MALDI was first pioneered by Karas and Hillenkamp in the 1980’s and was considered to be the most useful technique for ionizing large intact proteins greater than 10,000 Da.\textsuperscript{20} The analyte is incorporated into an organic matrix, which is exposed to laser radiation. It can be performed in a vacuum of less than $10^{-6}$ torr. A variety of matrices are used, including cinnamic acid, glycerol etc. UV or IR lasers are used for desorbing the neutral and charged analyte molecules from the sample matrix. Originally, the mass spectra were obtained by moving the laser across the sample in a raster pattern followed by generation of the chemical images. Recently studies using microscope mode in MALDI MSI have also been demonstrated.\textsuperscript{21} Several approaches have been described for deposition of matrix for tissues, including electrospray deposition\textsuperscript{5}, and spray nebulizer assisted deposition.\textsuperscript{22}

MALDI as an imaging technique has numerous applications. Peptides and proteins have been imaged using MALDI-MSI.\textsuperscript{5} MALDI has enabled chemical imaging of normal and cancerous tissues.\textsuperscript{23} Peptide distributions in invertebrate neurons have been imaged using MALDI.\textsuperscript{24} MALDI MSI has proven to be an important tool for detection of biomarkers for a variety of diseases, such as cancer\textsuperscript{25}, Alzheimer’s\textsuperscript{25, 26} and Parkinson’s disease.\textsuperscript{11} The functions of lipids in biochemical pathways in correlation to tissue histology have been explored using MALDI-MSI.\textsuperscript{14}
Figure 1.2. Schematic representation of MALDI.
1.3. Difficulties in Imaging Mass Spectrometry

1.3.1. Resolution and Sensitivity

The lateral resolution of a system determines the number of ions produced per pixel. Therefore at high lateral resolution there is a significant decrease in sensitivity. In MALDI, pulsed lasers are used to desorb and ionize the sample. MALDI typically requires the use of a laser spot size of greater than 10 \( \mu \text{m} \) to provide sufficient secondary ion signal. The minimum spot size of the laser depends on the diffraction limit, which in turn limits the lateral resolution. Therefore, the resolution less than 1 \( \mu \text{m} \) is difficult to achieve. Better resolution can be achieved with SIMS because a high-energy ion beam can be focused to a smaller spot than a laser beam. The use of SIMS for biological sample analysis was limited because of the insufficient sensitivity and resolution. The low amounts of individual components in biological samples result in low signals. Also, unfortunately, a large number of species are produced as neutrals thereby limiting the detection and imaging of low abundance molecules. The compression of the primary ion beam in SIMS to increase the lateral resolution results in a lower number of ions produced, leading to a trade-off between the resolution and sensitivity. Lateral resolution of 50-100 nm has been achieved by NanoSIMS these days with the use of reactive primary ions, which enhances the sensitivity. It allows simultaneous imaging of 5-7 secondary ions in parallel, generating precise maps of several different fragments at the same location. The use of surface treatment methods like Metal Assisted (MetA) SIMS and Matrix Enhanced (ME) SIMS have led to increased sensitivity\(^{27} \) and maximum retrieval of information from the tissue samples\(^{28,29} \) by enhancing the desorption and ionization of analytes.
1.3.2. Matrix Effects

In SIMS, secondary ion yield can vary over several orders of magnitude with changes in the chemical environment of the sample from one matrix to another. For example, the secondary ion yield of Al$^+$ from Al metal and Al$_2$O$_3$ differs by a factor of 100. Thus SIMS does not give quantitative data for the sample components. By the use of appropriate references, the absolute concentration could be determined. The yield of the secondary ions can be enhanced by the use of reactive primary ions: Cs$^+$ enhances the production of negative secondary ions, while O$_2^+$ results in enhanced positive secondary ion yield. MALDI has rarely been used for the analysis of low molecular weight sample analytes because a number of matrix ion peaks are seen in the low m/z region.

1.3.3. Vacuum

The removal of the bulk water is necessary because water can result in background peaks in the mass spectra. Therefore, it remains a challenge to preserve sample integrity while removing excess water. In addition to this, it is also important that the sample molecules do not sublime off the surface in ultrahigh vacuum. Different methods have been adopted to achieve these goals namely, fast freezing and low temperature dehydration. Others include ultramicrotomy, chemical fixation, and air drying.
1.3.4. Ion Suppression

Ion suppression is a phenomenon in which there is preferred ionization of some desorbed species, making them appear to be at high abundance in the mass spectrum, as opposed to other species, which can appear to be in low abundance in the secondary ion beam relative to their actual abundance in the sample. The relative intensity of the same protein in two different samples could be compared, but the relative intensities of two different proteins in the same sample could not be compared because of different ionization efficiencies. The optimization of sample preparation can lead to minimizing of the ion suppression. For example, the salts and lipids responsible for ion suppression of informative proteins in tissue sections are removed by using several steps of alcohol washing.\(^{29}\)

1.4. Imaging Mass Spectrometry under Ambient Conditions

Ambient ionization can be described as a technique in which the ionization of the sample occurs in the open environment, not inside the mass spectrometer, and the analyte ions are introduced into the mass spectrometer. It allows the untreated samples to be ionized in the ambient environment, and little to no sample preparation is required.\(^{36}\) This results in rapid analysis and high throughput.

Desorption electrospray ionization was the first ambient ionization technique. It was introduced in 2004.\(^{7}\) Since then, many other ambient ionization techniques has been developed, such as Direct Analysis in Real Time (DART)\(^ {37}\), Desorption Atmospheric Pressure Photolionization (DAPPI), Laser Ablation ElectroSpray Ionization (LAESI)\(^ {38, 39}\), Laser Ablation Flowing Atmospheric Pressure Afterglow (LA-FAPA)\(^ {40}\), and Low-Temperature Plasma (LTP).\(^ {41}\)
These techniques do not require a vacuum system for ionization and can be operated in the open, ambient environment, which makes them suitable for use outside the laboratory, in clinical or forensic settings, for example.

LAESI, like DESI, is a commonly-used soft ionization technique. In LAESI, a laser is used for desorbing the analytes from the sample surface, which are then ionized by collision with charged droplets produced by ESI. Other techniques that closely resemble LAESI are Electrospray Laser Desorption Ionization (ELDI) and Laser Electrospray Mass Spectrometry (LEMS). The only difference in these techniques is the use of different wavelength lasers i.e. femtosecond near-IR lasers for LEMS and nanosecond mid-IR and UV lasers for LAESI and ELDI. The applications of LAESI include imaging and identification of metabolites in plant tissues, analysis of rat brain tissues.
Figure 1.3. Schematic representation of LAESI.
Some other ambient ionization techniques, which are not as popular as DESI and were developed in the last decade, are now seeing various MSI applications. Atmospheric Pressure Femtosecond Laser Desorption Ionization (AP fs-LDI)\textsuperscript{45} is one of those. It uses a focused NIR femtosecond laser for ablation and ionization of the sample, which requires little to no sample preparation. It has been used for the analysis of onion epidermis. Its imaging capabilities were demonstrated by mapping the glucose ion found in onion tissue. Another ambient ionization technique, Infrared Laser Ablation Metastable-Induced Chemical Ionization (IR-LAMICI)\textsuperscript{46}, uses laser ablation for desorbing analytes and a plasma for chemical ionization. It has been used to analyze counterfeit tablets and tissue samples.

Plasma based ambient ionization sources include: Plasma Assisted Desorption Ionization (PADI), Low Temperature Plasma (LTP)\textsuperscript{47} and Laser Ablation coupled to Flowing Atmospheric-Pressure Afterglow (LA-FAPA).\textsuperscript{48} In the LTP, helium gas is used to produce a microplasma with a dielectric barrier discharge, which is then pointed onto a sample. It has been used to analyze bulk aqueous solutions as well as minute food samples for melamine contamination. In LA-FAPA, a UV laser beam is used for ablating the sample, which is then transferred in a nitrogen stream to a helium flowing atmospheric-pressure afterglow ionization source. For demonstrating imaging capabilities a logo was printed with caffeine doped ink and mapped. Another ambient plasma based ionization technique, Desorption Atmospheric Pressure Photoionization (DAPPI)\textsuperscript{49} uses heated solvents such as toluene or acetone for sample desorption. The desorbed sample is then ionized by a photoionization lamp producing 10 eV photons. DAPPI can be used with neutral and non-polar compounds. Samples that are susceptible to thermal degradation cannot be used with this technique. Applications include MSI of brain tissues and DAPPI-MS on a sage leaf.
In total about thirty ambient ionization techniques have been developed in the last decade. All techniques have some advantages as well as some disadvantages. Recent advances in instrumentation, sample preparation and a better understanding of their mechanisms have led to improved sensitivity and better resolution, and hence improvement in the overall capabilities of mass spectrometry.

1.5. **DESI Mass Spectrometry Imaging**

DESI Imaging is a variation of DESI that has the ability to record spatial and molecular information simultaneously on surfaces. It is done by placing a sample on a glass slide that is scanned in the X and Y directions in a 2-D array of predefined points. Molecular images are then constructed from one or more of the ion signals derived from the surface as a function of position in the array.

The mechanism for the ionization of analyte in DESI is an active area of investigation. Several mechanisms have been proposed, of which the droplet pickup mechanism is the most widely accepted. In droplet pickup the primary charged liquid droplets and the gas jet from a pneumatically assisted ElectroSpray (ES) ion source are directed at the surface to be analyzed. Impact of the primary droplets with the sample surface creates a thin liquid layer on the surface. Within this liquid layer dissolution and extraction of compounds at or near the sample surface occurs. The continued impact of primary charged droplets causes splashing of the liquid layer, ejecting secondary charged droplets containing the dissolved analyte. The droplets are drawn into the mass spectrometer via an ion transfer line.
Figure 1.4. Schematic representation of DESI desorption and ionization process.
1.5.1. Advances in DESI Imaging

In 2006 Cooks’ group published the first 2D imaging paper using DESI-MSI. In that paper they successfully mapped 2D images printed on a photographic paper with different inks and lipid distribution in the coronal section of rat brain. They reported lateral resolution of 400 µm for tissue experiments and 200 µm for the ink experiments. Since then the same group has published many papers on tissue mapping using DESI Imaging.

At the time Cooks group invented DESI Imaging and used it for tissue imaging, Van Berkel’s group was working on improving image resolution in DESI-MSI. They investigated imaging resolution using DESI-MSI on printed photographic paper and Thin Layer Chromatographic (TLC) plate surfaces. They reported a resolution of 40 µm, which was much better than the ones reported previously by other groups. They were able to achieve this resolution by carefully controlling certain parameters, such as spray-tip-to-surface distance, solvent flow rates, and spacing of lane scans.12

In another paper Van Berkel’s group reported the effects of various parameters, such as raster and unidirectional scanning, constancy of spray-tip-to-surface, and atmospheric sampling interface capillary-to-surface distances, on the molecular image quality using DESI-MSI.6 They determined that unidirectional scanning consistently gave better results than raster scanning in terms of spatial resolution and the quality of molecular images generated of the samples. They also found that constancy of spray-tip-to-surface and atmospheric sampling interface capillary-to-surface distances are essential to have accurate correlations of the concentrations on the surface to its image. It was also found that when the surface was tilted at an angle of 1.35° to the spray-tip there was big loss in resolution.6
In 2010, Teffera et al. described the effects of using heated nebulizing gas and acquiring high-resolution, accurate mass spectra on the chemical images of rat brain and liver tissues. They found that better images were obtained using high-resolution mass spectra, as the interfering ions were successfully avoided. Heated nebulizing gas caused increased ionization of the sample at lower nebulizing gas (nitrogen) pressure.50

In 2012 Cooks’ group published a paper on tissue imaging in which they were able to get spatial resolution as good as 35 μm, which is the best resolution reported till now with DESI-MSI under controlled experimental conditions.51 For confirming the said resolution, they imaged the known morphological features of the mouse brain tissues containing phospholipids. To achieve this resolution, parameters such as solvent composition, diameter of the emitter capillary, scan speed and solvent flow rate were carefully chosen.

1.5.2. Applications

The Cooks’ group first exploited the potential of DESI imaging apart from detecting dyes on photographic paper by detecting various lipids in rat brain tissues. Since then it has been used in various fields, including biological tissue analysis9, 17, 43, 52-60, thin layer chromatography separation and DESI analysis36, 57, 61-66, forensics55, 67-69, natural products imaging70-72 and reactive DESI imaging.45, 73-80

1.5.2.1. Tissue Analysis

The Cooks’ group was the first to demonstrate tissue imaging by DESI.81 They were able to resolve the spatial distribution of two lipids in the coronal section of rat brain. The images were
produced of the two lipids and correlations were drawn between the two based on the position and type of tissue. The reported resolution was 500 μm for this experiment, which was much lower than that produced by MALDI or SIMS. The advantages of ambient operation and minimal sample preparation outweighed the low resolution.

In another paper the same group was able to develop a method to make a rapid distinction between cancerous and non-neoplastic tissues using DESI-MSI. The distinctions were made on the basis of differences between lipid profiles of the cancerous human prostrate tissues and normal tissues. Cholesterol sulphate was found to be the distinguishing compound, which was found to be exclusively present in the cancerous tissues.

In early 2012, the Laskin group published a paper on tissue imaging using nano-DESI-MSI and reported a resolution of 12 μm, which is the best resolution reported by any group till now and is at par with other techniques such as MALDI. The nano-DESI is a variant of DESI that uses minute amounts of solvent positioned by surface tension between two capillaries comprising the nano-DESI source and the solid analyte. This configuration helps in controlled desorption of analytes on the substrate followed by ionization through self-aspirating nanospray.

1.5.2.2. Thin Layer Chromatography

DESI-MSI serves as a suitable detection technique for TLC plates because some compounds separated on TLC plates are not fluorescent and can’t be detected with UV-Vis spectroscopy. With DESI-MSI the molecular information as well as the spatial distribution of the compounds separated on TLC plates can be detected, and this makes DESI-MSI a powerful tool for detection.
In 2006, the Van Berkel group published a paper to illustrate the practical application of DESI-MSI in the field of TLC plates. They were able to image rhodamine dyes separated on TLC plates. DESI-MSI has also been used to map separated lipids mixtures. DESI-MSI has also been reported for the analysis of compounds like alkaloids and peptides when coupled with TLC separations.

1.5.2.3. Natural Products Imaging

Lane et al. were the first to image natural products present in tropical seaweed *callophycus serratus* by DESI-MSI. An imprint technique has been used for DESI-MSI as well. This technique is applied when the sample surfaces are rough and soft and DESI cannot be performed on the sample. The chemical species present on those surfaces are first transferred to a hard surface and then MS analysis is performed. Two applications include imprinting of plant tissue on photographic paper prior to its analysis and imprinting of bacterial metabolites on cellulose filter ester membranes.

1.5.3. Limitations

DESI-MSI has two major limitations. It has not been used to study low abundance compounds, although results on highly abundant compounds have been widely reported. The spatial resolution produced is approximately 250 µm under normal conditions, which is poorer than that produced by MALDI (5 µm – 200 µm) or SIMS (approx. 100 nm).
1.6. **Research Objective**

This thesis focuses on the design and optimization of the setup for performing DESI-MS imaging on various substrates. The proposed setup was tested for its lateral spatial resolution. To provide proof-of-concept of the design, preliminary tests were performed to generate images from commercial thin layer chromatographic plates and photographic paper. Chapter 3 focuses on the separation of dyes and analgesics on microfabricated Thin Layer Chromatography plates (M-TLC plates) and detection with DESI-MSI.
1.7. References


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Chapter 2. Desorption Electrospray Ionization Mass Spectrometry Imaging: Instrumentation, Optimization and Capabilities

2.1. Introduction

Desorption ElectroSpray Ionization (DESI), introduced in 2004, was the first ambient ionization technique. In DESI, charged liquid droplets along with a gas jet from a pneumatically assisted ElectroSpray (ES) ion source are directed at a target surface. On impact with the surface, these liquid droplets create a thin layer that dissolves and extracts compounds present at and/or near the surface. The continued impact of primary charged droplets causes splashing of the liquid layer, ejecting the secondary charged droplets containing the dissolved analytes. The secondary droplets then undergo an ESI like fission mechanism to produce gas-phase ions that are drawn into a mass spectrometer via an ion transfer line, and the mass spectra are recorded.

The possibility of ambient mass spectrometric analysis of almost any surface is the main reason for widespread interest in DESI-MS, as demonstrated by an increase in the number of studies published on this technique. The applications include forensic analysis of inks, probing marine natural product defenses, screening of counterfeit pharmaceutical tablets, detection of contaminants in food, biological tissue imaging, imaging of drugs and metabolites in tissues and urine, metabolomic studies of bacterial samples, detection of explosives, natural products imaging, 2D TLC separation and DESI imaging.
2.1.1. Goals

This chapter focuses on the design and optimization of the setup for performing DESI-MS imaging on various substrates. The proposed setup was tested for its lateral spatial resolution. To provide proof-of-concept of the design, preliminary tests were performed to generate images from commercial thin layer chromatographic plates and photographic paper.

2.2. Hardware Design

2.2.1. DESI source

2.2.1.1. Design Considerations

Our DESI source was based on the design proposed by Cooks’ group for their ESSI source, which uses 1/16” stainless steel Swagelok™ fittings. This design is functionally identical to Prosolia’s commercial DESI ion source and other home built DESI emitters, and has been tried and tested by many research groups.

The spray capillary of the DESI ion source is mounted just above the inlet capillary of the mass spectrometer and sample surface. The DESI source can be moved in the X, Y and Z directions for adjusting its distance from the sample and inlet capillary and controlling the spray angle above the sample. The sample is fixed on the movable stage and can be easily switched without compromising the optimized position of DESI ion source.

Since the automatic stage on which we secure our sample is mounted on an inverted microscope, some design modifications were necessary. The DESI source cannot be in contact with the stage, as the stage’s movement in the X and Y directions is necessary for scanning the
entire sample surface. This means that the DESI source must extend over the microscope stage on an arm bar that is attached to the ‘breadboard’ table on which the microscope is mounted. A rotational stage and an X, Y and Z stage were necessary to control the spray angle and position of the DESI ion source, respectively, relative to the microscope stage. The setup was described in detail in Michael Wood’s dissertation.33

The X-axis was the direction along which the DESI source was pointing when it was parallel to the microscope stage. The Y-axis was parallel to the rotational axis of the rotational stage, aiding the movement of DESI capillary in the XZ plane.

2.2.2. **Mass Spectrometer**

2.2.2.1. **Design Consideration**

For the DESI MS Imaging project, we purchased Bruker Daltonic’s micrOTOF Q mass spectrometer (Bruker Daltonics, Billerica, MA). There were various reasons for buying a time-of-flight mass spectrometer. First, TOF mass spectrometers have been successfully used for most IMS studies. Secondly, it has an open interface for sampling that can be easily coupled with a DESI setup for imaging.

The mass spectrometer required additional hardware, including a capillary extension, a capillary bridge support, a transfer line, a capillary clamp, and mounting hardware for this whole setup on the rail system so that it would be at the same height as the microscope and DESI source. The mount for the mass spectrometer incorporates a rotational stage and bearings for ease of replacement of the ion source and maintenance of the mass spectrometer. To perform mass analysis, the transfer line was extended over the microscope stage by elevating the mass
spectrometer to an appropriate height. The mount for the mass spectrometer was originally constructed for use with an LCQ ion trap instrument (Thermo-Fisher, Waltham, MA). It was adapted for the Bruker instrument using four feet of the design shown in Fig. 2.1.
Figure 2.1. Mass spectrometer stage foot.
2.2.2.2. **Transfer Line Setup**

The Bruker Daltonic’s micrOTOF II mass spectrometer came with an ESI source that needed to be replaced with custom built parts to sample analytes over the microscope. The capillary Extension assembly consists of three parts: a capillary extension, an extension tube and a capillary bridge support. The complete assembly is shown in figures 2.2 and 2.3.

Figure 2.2. Transfer line setup.
Figure 2.3. Transfer line setup side view.
2.2.2.3. Capillary Extension and Extension Tube

The capillary extension is shown in Figure 2.4. It is made of stainless steel and designed to slide over the glass inlet capillary of the mass spectrometer. It has three grooves at the back end and one hole in the front in which the transfer line can be inserted as shown in figure 2.4. A heat-resistant O-ring, placed in the second groove, was used to make an airtight seal between the heated glass capillary and capillary extension. The transfer line was charged by conducting voltage from the mass spectrometer inlet glass capillary to the capillary extension. This conduction of voltage was achieved by coiled springs placed in the first and third grooves of the capillary extension.

Figure 2.4. Capillary extension.
The extension tube is shown in figure 2.5. It is an 8-inch long stainless steel capillary tube with an 18° bend in its front end. This angle is called $\beta$ and is critical for imaging.

Figure 2.5. Extension tube.
2.2.2.4. Capillary Bridge Support, Gas Shield and Interlock Override

The capillary bridge support is shown in figures 2.6, 2.7 and 2.8. It has two stainless steel slide bars that can be inserted into two cavities already present on the face of the mass spectrometer. It also has a hole at the top end in which the front end of the capillary extension can fit. It helps to secure the capillary extension onto the glass capillary as shown in figure 2.2. Since the extension tube is at high voltage the inside of the top hole is coated with delrin, a nonconducting material.

![Figure 2.6. Capillary bridge support.](image)

<table>
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<tr>
<th>ITEM NO.</th>
<th>DESCRIPTION</th>
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</tr>
</thead>
<tbody>
<tr>
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<td>Capillary Bridge Support</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>Slide Bar</td>
<td>2</td>
</tr>
</tbody>
</table>
Figure 2.7. Capillary bridge support slide bar.
Figure 2.8. Capillary bridge support T.
The gas shield, shown in Figure 2.9, is made of ceramic. It gently slides over the mouth of the mass spectrometer and is secured in place with a screw. It directs the flow of heated nitrogen coming out of the mass spectrometer over the transfer line. This constant flow of hot nitrogen heats the transfer line.

Figure 2.9. Gas shield.
2.2.2.5. Interlock Override

There is a safety interlock switch on the mass spectrometer that needs to be pressed at all times for it to work. The interlock override is secured at the bottom right screw of the mass spectrometer face. With the interlock overridden there are exposed high voltages on the capillary extension, and care must be taken to switch off the voltages manually from within the software before touching the extension. The interlock override is shown in Figure 2.10.
Figure 2.10. Interlock override.
2.2.2.6. Capillary Clamp

The complete capillary clamp assembly is shown in figures 2.11 and 2.12. Except for the clamp, which is made of delrin, the rest of the assembly is made of an aluminum alloy and stainless steel. Delrin being a non-conductor prevents conduction of electricity from the transfer line to the microscope. The capillary clamp attaches to the microscope with the help of two screws. It is designed to precisely position the capillary extension over the sample. It helps the movement of the capillary extension tube in the Y and Z direction. For adjustments in the X direction we move our Mass Spectrometer. The clamp also prevents the wobbling of the capillary due to the gas and other issues.
Figure 2.11. Capillary clamp.
Figure 2.12. Capillary clamp top and front view.
2.3. **Experimental**

2.3.1. **Materials and Reagents**

HPLC grade methanol was purchased from Sigma Aldrich (St. Louis, MO, USA). Glossy photographic paper was purchased from Hewlett Packard and silica gel TLC plates were purchased from Merck KGaA (Darmstadt, Germany). An ExtraFine Sharpie (Stanford Corporation, Oak Brook, IL) was used to write on these surfaces. Basic Blue 7 dye was obtained from Sigma-Aldrich Corp (St. Louis, Mo, USA).

2.3.2. **Sample Preparation**

An ink jet printer was used to print parallel lines on the glossy photographic paper to measure the spatial resolution of the DESI-MSI experiment. (See figure 2.13) A black ink cartridge was doped with Basic Blue 7 dye that gave an intact cation signal at m/z 478.5. The name of our university, the ‘BYU’ symbol was written on a silica gel TLC plate and the letter ‘m’ was written on photographic paper using a purple sharpie. The line from the sharpie gave an intact cation signal at m/z 443.5, as rhodamine B is the principle dye in it. Methanol was used as the spray solvent for all experiments.

2.3.3. **Automated Sampling**

The H117 ProScan Flat Top Inverted Microscope X-Y robotic Stage equipped with H224XRLP specimen holder for the Nikon Microscope (Prior Scientific Inc., Rockland, MA) was used to hold TLC plates and other samples to be scanned relative to the stationary DESI ion source.
The stage was connected to a ProScan II controller (Prior Scientific Inc., Rockland, MA), which in turn was connected to a computer via a USB port. An in-house written MATLAB program was used for (i) controlling the mass spectrometer and (ii) manipulating the stage movement in the X and Y directions. The same MATLAB program was used to automate the entire process. The stage can also be operated manually with the help of a joystick. For initial positioning the stage was moved manually in the XY plane and the DESI source was positioned in the Z-axis over the stage. The incident angle of the DESI ion source was kept at 55°, 2.8 mm above the sample surface. Nitrogen was used as the nebulizing gas at 135 psi. Methanol at 1.5 μL/min was used as the spray solvent for spatial resolution experiments and imaging the letter ‘m’; and at 3 μL/min for imaging the ‘BYU’ symbol.

2.3.4. DESI-MS Imaging Analysis

All of the experiments were carried out on the micrOTOF II mass spectrometer (Bruker Daltonics, Billerica, MA) equipped with a custom-built inlet and a DESI ion source, which is described above in detail. The mass spectrometer was operated in positive ion mode and mass spectra were acquired from 400 m/z to 500 m/z. The spectra acquisition rate was set at 1 Hz and a rolling average of 1 was used. For the spatial resolution experiment, each lane was scanned at 112 μm/s. Unidirectional scanning was done for all the experiments.

2.3.5. Software

MSconvert (freeware, www.ProteoWizard.com) was first used to convert .BAF files generated from Bruker micrOTOF control version 3.0 to mzml files. These mzml files were then
converted to imzML files using imzML converter (freeware, www.maldi-msi.org). Finally, Biomap (freeware, www.maldi-msi.org) image analysis software was used to generate and visualize two-dimensional images of the surface.

2.4. Results and Discussion

2.4.1. Spatial Resolution

Spatial resolution in a DESI-MS image experiment can be defined as the size of the smallest feature distinguishable in a molecular ion image. The parameters that affect the spot size of the DESI spray plume and hence the resolution are solvent composition, solvent flow rate, inner diameter of the capillary tip, nebulizing gas flow rate, atmospheric conditions, surface material composition, diameter of the mass spectrometer transfer line orifice, scanning mode and imaging scan rate.

To analyze the spatial resolution of the DESI source to be used, the black ink cartridge of the printer was doped with basic blue 7 dye and patterns were printed onto a photographic paper. The pattern consisted of ‘lines’ having different widths, all having a 1 mm pitch. *Pitch* is defined as the distance between two consecutive ‘lines’ measured from their centers. Having a constant pitch, the dead space between them decreased with increasing line width. Various lanes were scanned on the pattern as shown in figure 2.13, each having different ‘line’ widths (lane 1 = 100 µm, lane 2 = 176 µm, lane 3 = 264 µm, lane 4 = 529 µm, lane 5 = 700 µm). The dead space (d) could be calculated by using the following formula:

\[ d = p - w \quad (1) \]

Where, \( p \) = pitch and \( w \) = width of ‘line’
The calculated dead space between the ‘lines’ of lane 1 was 900 µm and that of lane 5 was 300 µm. The experiments were performed by scanning one lane at a time at the rate of 112 µm/s in the direction as shown in the figure 16. The peak at m/z 478.5 represents the intact cation of the basic blue 7 dye. Scanning of each lane was done in single ion monitoring mode and peak intensities were plotted as a function of time as shown in figure 2.14.
Figure 2.13. Ink patterns printed on glossy photographic paper to check lateral spatial resolution. Each line represents the trajectory of the lane scanning. The ink was doped with basic blue 7 giving a peak at m/z 478.3.
Figure 2.14. Single ion monitoring mode chromatogram produced from scanning each successive lane for peak at m/z 478.5.
In figure 2.14, lane one is baseline separated with no “carry-over” effects. Lanes 2-4 are also baseline separated but the peak profile changes from roughly Gaussian to a plateau shape. A plateau is observed when the width of the line roughly equals the width of the spot. In this experiment it starts between 176 \( \mu m \) to 264 \( \mu m \). At this moment, the resolution was estimated to be ca. 200 - 250 \( \mu m \). To prove this assumption, I tried to resolve 800 \( \mu m \) and 750 \( \mu m \) lines, both having a pitch of 1 mm, without any success. The dead space was 200 and 250 \( \mu m \) respectively. Of the peaks in the figure 2.14, for 700 \( \mu m \) line widths, only one pair is baseline separated. The non-zero baseline between the peaks was attributed to the carry-over between the lines due to ink bleeding. The bleeding of the ink was visible to the naked eye. Therefore, the resolution was determined to be ca. 250 -300 \( \mu m \).

The serrated tops of peaks as seen in some of the lanes are due to the electronic and ion statistical noise. The same experiment was done at different solvent flow rates and stage speed, and it was found that the spatial resolution decreased at higher solvent flow rate (see figure 2.15).
Figure 2.15. Single ion monitoring mode chromatogram produced from scanning each successive lane for the peak at m/z 478.5.
The decrease in resolution at higher solvent flow rates is due to the increased flux of impacting droplets and increased size of impact region. The high flow rate also caused excessive sample wetting, dilution and redistribution of analytes on the surface. Figure 2.16 (not drawn to scale) shows schematic representation of the spot profile of DESI spray at different solvent flow rates.

Figure 2.16. Shematic representation of DESI spray spot profile (not drawn to scale).
2.5. **DESI-MS Imaging**

DESI Imaging is a variation of DESI and it has the ability to simultaneously capture spatial and molecular information on surfaces. Imaging is done by placing a sample on a glass slide and scanning the sample in the X and Y directions in a 2-D array of predefined points. Molecular images are constructed from one or more of the ion signals derived from the surface as a function of the position in the array.

To check the effectiveness of our setup for performing DESI-MSI studies, I imaged photographic paper and silica gel TLC plates. A purple sharpie, which has a rhodamine B dye base, was used to write the letter 'm' (1 cm x 0.76 cm) and the 'BYU' symbol (1.7 cm x 0.78 cm) on photographic paper and a silica gel TLC plate respectively. For imaging the letter ‘m’ on photographic paper, each lane was scanned continuously 1 cm along the X-axis, at a surface speed of 112 μm/s and in steps of 200 μm along the Y-axis. The total area scanned was 7.6 x 10^7 μm^2 producing an array of 90 x 38 (3520 pixels). Figure 2.17 shows the optical image, molecular ion image, direction of scan and positive ion DESI mass spectrum of rhodamine B dye at m/z 443.3. As it is evident from the pictures that the molecular ion image looks exactly like the optical image and that the features can be correlated to such an extent that we could superimpose the two images. The curvature in the letter ‘m’ is also resolved so that we can say there are no carryover effects.
Figure 2.17. A) Molecular ion image of the letter “m” written on glossy photographic paper recorded by DESI-MSI. B) Optical image of the letter “m” written on glossy photographic paper. C) Black arrows followed by blue arrows in the figure show the route of the probe during automated lane scanning of the photographic paper. D) Positive ion DESI mass spectrum of the purple sharpie that has rhodamine B based dye showing a peak at m/z 443.3.
One of the aims of my work was to explore the potential of DESI-MSI for the detection of analytes on TLC plates. Therefore, checking the feasibility of our DESI-MS imaging setup on TLC plates was critical. ‘BYU’ was written on a commercial silica gel TLC plate, which was used for preliminary testing. For imaging the ‘BYU’ symbol (1.7 cm x 0.78 cm) on a TLC plate, each lane was scanned continuously 1.7 cm along the X-axis, at a surface speed of 112 μm/sec, and each step along the Y-axis was 300 μm. The total area scanned was approx. $1.3 \times 10^7 \text{μm}^2$ producing 150 x 26 (3900 pixels). Figure 2.18 shows the optical image, molecular ion image, and direction of scan and positive ion DESI mass spectrum of rhodamine B dye at m/z 443.3. The optical and molecular ion images are in good accordance with each other, although there is some variation in signal intensity across the image. This might be due to variations in dye concentration. Overall, there is good correlation between images, which suggests that the written features can be successfully resolved on TLC plates using DESI-MS imaging. This also provides promise for detecting real world analytes separated on a TLC plate (discussed in Chapter 3).
Figure 2.18. A) Molecular ion image of the “BYU” symbol written on a silica gel TLC plate recorded by DESI-MSI. B) Optical image of the “BYU” symbol written on silica gel TLC plate. C) Black arrows followed by blue arrows in the figure show the route of the probe during automated lane scanning of the photographic paper. D) Positive ion DESI mass spectrum of purple sharpie showing peak at m/z 443.3
Figure 2.19. Structures of analytes studied.

Basic Blue 7
m/z = 478.3

Rhodamine B
m/z = 443.2
2.6. Conclusions

The results presented here successfully demonstrate the ability of our setup to perform DESI-MS imaging on various surfaces. With DESI-MSI, the molecular information as well as the spatial distribution of the compounds separated on the surfaces can be recorded simultaneously, making DESI-MSI a powerful tool for detection. Automation of the process was achieved by an in–house written MATLAB program which controlled stage movement and mass spectrometer initiation through a TTL port. A spatial resolution of 300 μm was achieved, which can be further improved by using a low solvent flow rate and minimizing the DESI emitter tip-to-surface distance. The success of the present setup and proof-of-concept of DESI-MSI opens wide doors to further explore the potential of this technique on different surfaces with more challenging, real world analytes.
2.7. References


Chapter 3. Desorption Electrospray Ionization Mass Spectrometry Imaging on Microfabricated Thin Layer Chromatography Plates

3.1. Introduction

DESI (Desorption ElectroSpray Ionization) is the first ambient ionization technique developed by Cooks and coworkers at Purdue University. In DESI, a pneumatically assisted spray from the electrospray source is directed onto a surface, followed by desorption and gaseous ion formation of the analytes. The ions are then transferred via an orifice into the mass spectrometer and analyzed. Although, commonly used for the analysis of polar and ionic compounds, its use for the analysis of non-polar compounds has also been reported. DESI experiments are performed with minimal sample preparation, making it useful for high throughput analysis.

Although, the exact mechanism of DESI is still under investigation, the proposed mechanism involves the removal of the analyte molecules from the surface by charged droplets. It is a two-step process in which the primary droplets dissolve the analyte on impacting with the surface, followed by splashing on impact with the subsequent primary droplets, producing secondary microdroplets. Studies involving observation of solution-phase complexes between sprayed species and molecules on the surface and Doppler particle analysis also support a mechanism involving charged droplets containing the analyte. These studies showed the average droplet velocity and droplet diameter to be 150 m/s and 3 μm respectively. The soft ionization nature of DESI was confirmed by “survival ion yield” method, which showed the internal energy distribution of ions to be 2 eV. The spectra obtained from DESI analysis are dependent on a number of parameters, including solvent flow rate, emitter tip-to-surface distance, nature of the surface and spray solvent composition.
DESI has proven to be useful in the pharmaceutical industry, with high throughput analysis of tablets at a rate of 3 samples/s. A number of groups have employed DESI for various studies such as investigating ointments and drugs of abuse in tablets. DESI has also been used for the detection of residual explosives and chemical warfare agents. This method was very sensitive, with limits of detection ranging from picograms to femtograms for an array of compounds, including RDX (trinitrohexahydro-1,3,5-triazine), TNT (1,3,5-trinitrotoluene), and DMMP (dimethylmethylphosphonate). In combination with a field portable mass spectrometer, DESI has been used for in-field analyses of chemical warfare agents and environmental toxins. Using DESI, various phytochemicals/metabolites, including alkaloids, carbohydrates, lipids and amino acids were identified in direct tissue analysis of several plants namely, Atropa belladonna, Jimsonweed, Datura stramonium. Similarly, high throughput analysis on thin sections of animal tissues have been reported with the help of this technique. Spatial distributions of several tissue components e.g. membrane phospholipids have been studied using DESI imaging. Minimal sample preparation allows the analyses of various human specimens (e.g. urine, serum) for the detection of metabolites/biomarkers for several diseases as hundreds of samples can be analyzed in an hour. For example, analysis of tryptic fragments of equine cyt C using DESI has resulted in the detection and identification of 26 fragments. DESI has also been successfully used for the enantiomeric studies of chiral analytes, specifically for the quantitative analysis.

The ability of DESI to perform analyses directly from the surface within a few seconds allows the analysis of compounds directly from the stationary phase of a TLC plate. A number of surfaces, including polymethylmethacrylate (PMMA) and polytetrafluoroethylene (PTFE) and TLC plates, have been tested and evaluated in terms of their performance with DESI-MS. Prior to
detection of analytes by DESI, separation of complex mixtures can be carried out on TLC plates. TLC has been coupled to mass spectrometry successfully using DESI to study a number of hydrophobic and wettable stationary phases by optimization of different conditions, including solvent flow rate, solvent composition, nebulizing gas flow rate, DESI emitter-to-surface distance and chromatographic readout resolution.21

Separations of rhodamine dyes performed on reversed phase C8 and C2 TLC plates have been studied by combining TLC/DESI MS in positive ion mode using selected reaction monitoring on a hybrid triple quadrupole linear ion trap mass spectrometer. Also, the separation of FD and C dyes has been performed on wettable C18 plates in negative ion full scanning mode. A mixture of different components including caffeine, acetaminophen, and aspirin in a medicinal formulation was separated on a normal phase silica gel TLC plates and detected via DESI-MS in positive ion full scan mode.22

The detection of dyes was first carried out on a TLC plate with the help of a computer-controlled plate movement to scan a single development lane.22 Furthermore, modifications of sampling capillary of a commercial ES-MS instrument and of the software controlling the X/Y/Z stage sample holder (both made in-house) were made, which allowed multiple development lanes to be scanned on a TLC plate and imaging of analyte bands in a development lane.21

The microfabricated TLC plates (M-TLC) used for my experiments were assembled using carbon nanotubes (CNT) as the template upon which adsorbent materials could be precisely placed. The plates have superior chromatographic characteristics because the adsorbent bed is highly homogeneous. The patterned CNT forests were grown using chemical vapor deposition on a thin layer (~6 nm) of catalytic material (Fe). The CNTs were then coated with silicon nitride using a Low-Pressure Chemical Vapor Deposition (LPCVD) method. The coating step was
followed by CNT removal by heating at 1000 °C. This oxidation process burned off the CNTs and converted silicon nitride to silica. After oxidation, hydroxylation was done in an ammonium hydroxide bath at pH 10.0 to replenish silanol groups necessary for chromatography on the silica surface. The fabrication was done in similar fashion as described in figure 3.1.23-25

The purpose of these MS experiments was to demonstrate the compatibility of these novel M-TLC plates with DESI-MS imaging and its application to real-world samples. Analytes separated on M-TLC plates have previously being analyzed with UV light in case of fluorescent compounds 26 and with a densitometer in the case of non-fluorescent compounds.27 These new M-TLC plates have shown extremely fast separations and efficiencies in previous publications.23-25,28, 29 The plates are binderless, which implies that binder for adsorbent attachment to the substrate (e.g. glass, alumina etc.) is absent. These advantages give an edge to M-TLC plates over their conventional TLC and high pressure Thin Layer Chromatography (HPTLC) counterparts. DESI-MS can be useful in this technology to help identify separated analytes without using any conventional detection methods, such as densitometry or derivatization methods using different reagents.
Figure 3.1. Schematic of fabrication of M-TLC plates.
3.2. **Experimental Section**

3.2.1. **Materials.**

HPLC grade toluene, acetone and methanol were purchased from Fisher Scientific (Hampton, New Hampshire). The developing chamber and Linomat V were purchased from CAMAG (Muttenz, Switzerland). Phenacetin (≥98%, HPLC), propyphenazone, basic blue 7 and rhodamine B were purchased from Sigma-Aldrich Corp (St. Louis, Mo).

3.2.2. **Fluorescent Dye Separation and DESI-MSI**

Rhodamine B and basic blue 7 standard stock solutions (2mg/mL) were prepared in methanol. The working solutions were obtained by diluting the stock solutions to a concentration of 0.2 μg/μL. 0.5 μL aliquots of each dye solution were spotted at the same spot using the Linomat, making a 3-mm long sample band on the TLC plate. After spotting, the plate was dried on a hot plate at 120 °C for 15 s and then cooled to room temperature. The M-TLC plate was then placed in twin trough development chamber from CAMAG. The plate was equilibrated in the saturated chamber for a minute and developed in t-butylbenzene mobile phase for a distance of 25 mm. The run time on M-TLC plate was 1 min 28 s. Developed plates were dried on a hot plate at 120° C for 10 s before the DESI-MSI experiment. Cationic signals at m/z 443.3 and m/z 478.5 were obtained and plotted in traces that represented rhodamine B and basic blue 7, respectively.
3.2.3. Analgesics Separation and DESI-MS

Phenacetin (10 mg/mL) and propyphenazone (5 mg/mL) solutions were made in methanol. 3 μL of each solution was spotted on an M-TLC plate 0.5 mm from bottom using the Linomat to give a 3 mm long sample band. The M-TLC plate was then dried at 120 °C for 15 s and then cooled to room temperature. A development chamber was saturated with the mobile phase (toluene:acetone, 4:1, v/v) for ten minutes. The spotted plate was then kept inside the chamber and equilibrated for a minute. The plate was developed with 3 mL of mobile phase for a distance of 30 mm followed by drying on a hot plate (120 °C) to remove any solvent.
Figure 3.2. Structures of analytes studied.

Propyphenazone  
\[ m/z = 231 \]

Phenacetin  
\[ m/z = 180.2 \]

Basic Blue 7  
\[ m/z = 478.3 \]

Rhodamine B  
\[ m/z = 443.2 \]
3.2.4. DESI-MS Imaging Setup

Figure 1.4 shows the general layout of the DESI ion source and mass spectrometer inlet. The DESI ion source used for these experiments is similar to prototype design from Prosolia INC., (Indianapolis, IN) and is constructed in-house. The DESI ion source consisted of two concentric capillaries. The inner capillary (fused silica, 75 μm i.d., 150 μm o.d.) (Polymicro Technologies, AZ) delivers pure HPLC grade methanol and an annular outer capillary (fused silica, 250 μm i.d., 350 μm o.d) delivers the nebulizing gas. The two concentric capillaries were used to make the spray tip and were mounted using a Swagelok™ T-union in a way so that the inner capillary is protruding out of the outer capillary by approximately 0.2 mm. The protrusion is necessary to have a good solvent spray. The back of the inner capillary was connected to the 250 μL glass syringe mounted in the syringe pump after passing it from the back of the T-union. The outer metal end of the syringe pump was wrapped with copper tape, which was connected to the high power voltage supply at +4.9 kV through with an alligator clip. Nitrogen at 135 psi was used as the nebulizing gas. The incident angle was 55° and the spray tip-to-surface distance was kept at 2.8 mm for all experiments.

The mass spectrometer was a Bruker micrOTOF (Bruker Daltonics), which was operated using a micrOTOF control version 3.0 with custom built extensions and an X-Y robotic stage described in detail in Chapter 2. The mass spectra were acquired in positive ion mode.

3.2.5. Software

MSconvert (freeware, www.ProteoWizard.com) was first used to convert .BAF files generated from Bruker micrOTOF control version 3.0 to mzml files. The mzml files were then
converted to imzML files using imzML converter (freeware, www.maldi-msi.org). Biomap (freeware, www.maldi-msi.org) image analysis software was used to generate and visualize two-dimensional images of the surface.
Figure 3.3. DESI parameters.

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<tr>
<td>$\beta$</td>
<td>Collection angle</td>
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<tr>
<td>$d_1$</td>
<td>Spray tip-to-surface distance</td>
</tr>
<tr>
<td>$d_2$</td>
<td>MS inlet-to-surface distance</td>
</tr>
</tbody>
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3.3. Results and Discussion

3.3.1. Optimization of Analyte Signal

Prior to imaging experiments on M-TLC plates, the solvent flow rate was optimized to give high signal levels without loss of spatial resolution. For the optimization experiments, rhodamine B and BB7 dyes were spotted (3 mm bands) separately at the same spot on the M-TLC plates. The plates were then developed up to 25 mm. The developed analyte bands were scanned at 112 microns/sec under a stationery DESI emitter. Spray solvent flow rates of 3, 5, and 10 μL/min were used for scanning different lanes with methanol. The optimum spray solvent flow rate was found to be 3 μL/min for the M-TLC imaging experiment. The mass spectral signal levels at 3 μL/min and 5 μL/min are shown in figure 3.4. At low flow rates (~1 μL/min) there was no mass spectral signal. At 3 μL/min and 5 μL/min flow rate there was good mass spectral signal, which shows that high flow rate is needed for desorption ionization on M-TLC plates. A solvent flow rate of 10 μL/min resulted in complete erosion of the stationery phase from the M-TLC plate surface.
Figure 3.4. (A) Extracted ion chromatogram of BB7 and rhodamine B at 5 μL/min solvent flow rate.

(B) Extracted ion chromatogram of BB7 and rhodamine B at 3 μL/min solvent flow rate.
3.3.2. Lane Scan DESI-MS of Analgesics Separated on M-TLC plate

Lane scanning is a type of surface scanning method in which the TLC plate is scanned along the X-axis starting from the point where the analyte was spotted till the point where the TLC plate was developed. Lane scanning helps to determine all the analytes present in a lane, which are not easily discernable by visual inspection.

The data shown in figure 3.5 represent the lane-scanning mode of DESI-MS analysis. The X-axis represents the total lane scan distance (30 mm) starting from sample (analgesic) application spot till the solvent front. Figures 3.5A and 3.5B show the DESI-MS extracted ion chromatograms of phenacetin and propyphenazone respectively. It is clearly visible from the chromatograms that the separated analgesics peaks were obtained but were not baseline separated. This can be attributed to higher solvent flow rate or overloading sample application on M-TLC plate. The peak at m/z 180.2 in figure 3.5B represents phenacetin. Propyphenazone was clearly visible at m/z 231 in the relative intensity graph as seen in figure 3.5D. These peaks represent the protonated [M+H]+ ions of the molecules. Peaks at m/z 202 and m/z 217 are attributed to contaminants fouling the MS. It is important to note that peaks at m/z 180.2 and m/z 231.0, referring to phenacetin and propyphenazone respectively were not observed in the blank run. The retention of phenacetin is greater in the M-TLC plates than propyphenazone. This is explained by the fact that phenacetin has lower molecular mass than propyphenazone, and is more polar. The desorption efficiency of propyphenazone was found to be 10 x higher than that of phenacetin. A spray solvent rate of 7 μL/min was used to ensure adequate sensitivity for both compounds.
Figure 3.5. Detection with DESI-MS of the separated mixture of analytes on M-TLC plate. Concentration of analytes spotted are; phenacetin (10 mg/mL) and propyphenazone (5 mg/mL); injection volume 3 μL each; M-TLC mobile phase: toluene/acetone (4/1, v/v); elution distance: 30 mm; spray solvent: methanol; solvent flow rate: 7 μL/min. (A) Extracted ion chromatogram (EIC) of phenacetin. (B) Relative intensity graph of phenacetin. (C) Extracted ion chromatogram of propyphenazone. (D) Relative intensity graph of propyphenazone. The relative ion intensity mass spectrum shown in (B) and (D) were acquired from the highest spot on EIC peak maxima in (A) and (D) respectively.
3.3.3. Imaging of Analyte Bands Separated on M-TLC plate.

The lane-scanning mode can be utilized to map the entire TLC plate if multiple lanes are scanned successively. The distance between two consecutive lanes depends on the spatial resolution of the setup. In our case it was 300 µm. While scanning, we start with a corner of the spotted analyte mixture (3 mm band) and then surface scan the TLC plate on the X-axis. After this, the scanning probe was made to come back at starting position and then moved along the Y-axis, scanned again in X-axis and moved back to starting position. This step was repeated till we scanned the entire surface. Moving the stage back to the starting position helps prevent contaminating the part of the surface that is yet to be analyzed, as shown in 3.6D.

Figure 3.6 shows the optical image of a developed M-TLC plate prior to DESI analysis (part A), the selected m/z selected mass spectrometric image of BB7 and rhodamine B (part B and C, respectively), direction of scan (part D) and relative intensity graphs from two spots on the M-TLC plate for the dyes (part E and F). For imaging, the M-TLC plate was continuously scanned in the X direction in unidirectional scanning mode. Each lane in the X-axis was scanned at 112 µm/s and took 223 seconds to complete. Each step in the Y-axis was set at 300 µm because it is limited by the resolution of current set up. Methanol was used as the spray solvent at a flow rate of 3 µL/min. The total area scanned was 25 mm x 3.4 mm (85 mm²) producing an array of 112 x 12 (1344 pixels). The spectra rate and rolling average were set at 1 Hz and 2 respectively. The total analysis time was approximately 45 minutes. The molecular ion images generated were represented by the false color intensity scale with the relative ion intensities of the analytes represented by different color intensities. A red color in the images represents the highest intensity ions and black represents the lowest intensity ions.
DESI-MSI (Dyes) on M-TLC Plates

Figure 3.6. Detection with DESI-MSI of a separated mixture of analytes on an M-TLC plate. Concentration of analytes spotted: basic blue 7 (0.2 μg/μL) and rhodamine B (0.2 μg/μL); injection volume 3 μL each; M-TLC mobile phase: t-butylbenzene; elution distance: 25 mm; spray solvent: methanol; solvent flow rate: 3 μL/min. (A) Optical image of developed M-TLC plate. The blue spot and the red spot on the TLC plate are BB7 and rhodamine B respectively. (B) Molecular ion image of BB7 dye recorded by DESI. (C) Molecular ion image of rhodamine B recorded by DESI. (D) Black arrows followed by blue arrow show the route of the probe during automated lane scanning of the TLC plate. (E) Relative intensity graph of BB7 dye from a spot on the TLC plate. (F) Relative intensity graph of rhodamine B dye from a spot on the TLC plate.
3.4. Conclusions

We have demonstrated the compatibility of novel M-TLC plates with DESI-MS. Fast separations, and improved efficiencies are some of the advantages shown by these M-TLC plates. The potential of DESI-MS as a detection tool was studied. Baseline separation of two dyes (basic blue 7 and rhodamine B) was obtained on M-TLC plates and the analytes were imaged using DESI-MSI. These molecular ion images complemented the observed visual analyte bands. Unfortunately, these M-TLC plates didn’t have any fluorescent marker, which would make detection of non-fluorescent compound somewhat easier. The visual detection becomes further difficult if analytes lack chromophores. To portray the indispensable use of DESI-MS under these conditions, a pair of analgesics (phenacetin and propyphenazone) was separated on these plates. DESI-MS results showed two analyte peaks, but they were not baseline separated. These experiments also show the sensitivity of the M-TLC plates to DESI-MS. In the future, separated analytes can be analyzed using DESI imaging in addition to more traditional methods.
3.5. **References**


Chapter 4. Conclusions and Future Work

4.1. Conclusions

The main focus of my graduate work was to design and optimize a new set-up to perform desorption electrospray ionization mass spectrometry imaging. This concept was extended for analyte detection on microfabricated thin layer chromatographic plates. My first efforts were aimed at designing critical parts to aid hyphenation of DESI with the commercial MS equipment, which included a mass spectrometer stage foot, capillary extension, extension tube, capillary bridge support, gas shield and interlock override. The dimensions of these parts were carefully optimized based on literature precedent and experimental results. For increased robustness and reproducibility of the setup, it was necessary to fully automate the process. To aid this, a flat top inverted microscope X-Y robotic stage was employed and a MATLAB program was written in-house that could simultaneously control the robotic stage and mass spectrometer. We could achieve spatial resolution of 300 μm for our set-up, which was comparable to published results.

For preliminary experiments DESI-MSI was performed on commercial silica gel TLC plates and photographic paper. The generated molecular ion images were matched with their corresponding optical images, and features could be correlated to such an extent that we could superimpose the two images.

Dr. Linford’s group at BYU has developed novel M-TLC plates, which use patterned carbon nanotube templates to produce silica normal phase TLC plates, following a series of processes described in chapter 3. After the preliminary success with the DESI-MSI setup, imaging was done on these M-TLC plates to aid detection of an array of analytes. The separation of a dye mixture consisting of basic blue 7 and rhodamine B was imaged using DESI, which showed
baseline separation. These images were in correlation with the visual pattern on the M-TLC. Here, it is important to mention that the M-TLC plates are not fluorescent. Therefore, the potential of DESI-MSI could be exploited while using non-fluorescent analytes that are not visually detectable. To prove the utility of DESI-MS for use with non-fluorescent analytes, a mix of analgesics, consisting of propyphenazone and phenacetin was employed. The M-TLC plate was developed and lane scanning was performed to reveal two distinct peaks, which were not perfectly resolved. The two peaks correspond to the two analytes present in the mix, giving signals at characteristic m/z values. Lane scan DESI-MS mode shows promise in separating a single co-eluted sample band into its constituent analytes based on the fact that each analyte would have a peak at a specific m/z value.

4.2. Future Work

During my graduate work, I was able to design and optimize a successful setup for DESI-MSI and show its potential as an indispensable analytical tool. I was able to get a spatial resolution of 300 μm under normal operating conditions, which was comparable to published results. But some groups have reported spatial resolution of 40 μm under well-controlled specialized experimental conditions.1,2 The present setup needs to be further optimized to improve the spatial resolution under normal experimental conditions, to facilitate its widespread acceptance in the analytical community. This can be achieved by minimizing the emitter tip-to-surface distance, lowering the solvent flow rate and scan speed.

A few limitations related to high spatial resolution are low sensitivity and increased analysis time. At high spatial resolution, fewer analyte ions are generated because the area under
examination is small and fewer molecules are desorbed and ionized. To compensate for this, the number of scans should be increased, leading to increased analysis time. Instruments with high sensitivity would yield desirable signal even at faster analysis rate and hence can improve analysis time. Another approach by which the analysis time can be decreased is by using the microscope mode of imaging instead of microprobe mode of imaging. The microscope mode would come with a trade-off, requiring high vacuum and sophisticated optics for operation.

Another field of focus in the future should be “Reactive-DESI”. Here, certain additives are added along with a spray solvent that attacks the target analyte and forms ionic moieties that are easily detected by the MS with high sensitivity. For example, addition of 0.1% formic acid to the spray solvent helps in ionization of compounds that are otherwise not easily detectable by DESI. The most commonly used DESI spray solvents are pure methanol, a mixture of water and methanol, and a mixture of water and acetonitrile. Changes in spray solvent composition, such as addition of surfactants or using non-aqueous spray solvents, can also affect desorption/ionization capabilities and hence the information obtained.

Another improvement that can be made to DESI-MSI is to make it a quantitative technique. Currently, only the relative amount of the compounds interrogated from the surface can be determined. To make DESI-MSI a quantitative technique, a ‘standard addition’ approach could be used. An extrinsic compound could be homogenously added to the whole surface to be analyzed and data could be plotted as a ratio of absolute intensity of the compound under interrogation vs. ratio of absolute intensity of the extrinsic compound added. The extrinsic compound should be chosen carefully so that it will have similar chemical properties to the target analyte, like molecular structure, molecular weight, proton affinities and desorption/ionization efficiencies. Using this approach, only one or few out of numerous possible compounds under investigation can be
quantitated because the chemical properties of the ‘reference’ compound could be similar to few or even just one of the compounds. However this will require a significant amount of method development.
4.3. References

MATLAB Code for Controlling the XY Robotic Stage and Mass Spectrometer

```matlab
clear
response = 'empty string';
rresponse = " ";
s = serial('com4','BAUD',9600,'Terminator',13); fopen(s);

command{3} = 'SMS 62500 1';
command{1} = 'TTL 1 1';
command{2} = 'TTL 1 0';
command{4} = 'GR -70000 0';
%command{5} = 'TTL 0 1';
%command{6} = 'TTL 0 0';
%command{7} = 'SMS 100';
%command{8} = 'M';
%command{9} = 'GR 0 -400';

%tmtool this command is for deleting object created

CR = char(13);
LF = char(10);
% command = {command1,CR,command2,CR,command3,CR,command4};
% fprintf(s,command);
len = length(command);
% while response(1:len) ~= 'END'

for ii = 1:len
    disp(command{ii});
    try
        % because fprintf was giving error so used try and catch
        fprintf(s,command{ii});
    catch
        response = fscanf(s);
        rresponse = [rresponse,response];
    end
end

rresponse
fclose(s);
delete(s);
```

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List of Abbreviations

AP fs-LDI - Atmospheric Pressure Femtosecond Laser Desorption Ionization

BB7 – Basic Blue 7

C8 – Carbon 8

C18 – Carbon 18

CNT - Carbon NanoTubes

DESI - Desorption ElectroSpray Ionization

DART - Direct Analysis in Real Time

DAPPI - Desorption Atmospheric Pressure PhotoIonization

ELDI - Electrospray Laser Desorption Ionization

ES – ElectroSpray

EIC – Extracted Ion Chromatogram

HPTLC – High Pressure Thin Layer Chromatography

IR-LAMICI - Infrared Laser Ablation Metastable-Induced Chemical Ionization

IMS – Imaging Mass Spectrometry

ID – Inner Diameter

LAESI - Laser Ablation ElectroSpray Ionization

LA-FAPA - Laser Ablation Flowing Atmospheric Pressure Afterglow

LTP - Low-Temperature Plasma

LEMS - Laser Electrospray Mass Spectrometry

LA-FAPA – Laser Ablation coupled to Flowing Atmospheric-Pressure Afterglow

LPCVD - Low-Pressure Chemical Vapor Deposition

MSI - Mass Spectrometry Imaging
MALDI – Matrix-Assisted Laser Desorption Ionization
MALDI-MSI - Matrix Assisted Laser Desorption Ionization Mass Spectrometry Imaging
M-TLC – Microfabricated Thin Layer Chromatography
NIR – Near Infrared
OD – Outer Diameter
PADI - Plasma Assisted Desorption Ionization
PMMA - Polymethylmethacrylate
PTFE – Polytetrafluoroethylene
SIMS - Secondary Ion Mass Spectrometry
SIMS-MSI - Secondary Ion Mass Spectrometry Imaging
TOF-SIMS – Time-Of-Flight Secondary Ion Mass Spectrometry
TLC - Thin Layer Chromatography
TTL – Transistor-Transistor Logic
UV – Ultra Violet