APPLICATIONS OF NANOMANIPULATION COUPLED TO NANOSPRAY MASS SPECTROMETRY IN TRACE FIBER ANALYSIS AND CELLULAR LIPID ANALYSIS

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The novel instrumentation of nanomanipulation coupled to nanospray mass spectrometry and its applications are presented. The nanomanipulator has the resolution of 10nm step sizes allowing for specific fine movement used to probe and characterize objects of interest. Nanospray mass spectrometry only needs a minimum sample volume of 300nl and a minimum sample size of 300attograms to analyze an analyte making it the ideal instrument to couple to nanomanipulation. The nanomanipulator is mounted to an inverted microscope and consists of 4 nano-positioners; these nano-positioners hold end-effectors and other tools used for manipulation. This original coupling has been used to enhance the current abilities of cellular probing and trace fiber analysis. Experiments have been performed to demonstrate the functionality of this instrument and its capabilities. Histidine and caffeine have been sampled directly from single fibers and analyzed. Lipid bodies from cotton seeds have been sampled indirectly and analyzed. The few applications demonstrated are only the beginning of nanomanipulation coupled to nanospray mass spectrometry and the possible applications are numerous especially with the ability to design and fabricate new end-effectors with unique abilities. Future study will be done to further the applications in direct cellular probing including toxicology studies and organelle analysis of single cells. Further studies will be directed in forensic applications of this instrument including gunshot residue sampled from fibers.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

1.1 Introduction to Mass Spectrometry

The mass spectrometer separates and analyzes molecules based on their mass-to-charge ratio m/z. The periodic table gives the average molecular weight of an element, but a mass spectrometer measures the exact weight. With a mass spectrometer multiple peaks are observed with different isotopic distributions of each compound, used to identify compounds with the same molecular weight but different molecular formulas[1] . The isotopic distribution is formed by the different isotopes of a molecule; carbon has two naturally occurring isotopes carbon 12 with 99% natural abundance and carbon 13 with 1% natural abundance, so it is expected that a molecule with multiple carbon atoms to have a large peak with multiple carbon 12 atoms present and a small peak with many carbon 12 and a single carbon 13 present, shifting the mass by one. Some molecules that are similar, other than isomers, have the same molecular weight but different fragmentation patterns used to fingerprint molecules and identify them. Large libraries are available for many different compounds fragmentation patterns in order for ion identification. The ions are identified based on their m/z, isotopic distribution, and fragmentation patterns.

There are two running modes; a negative ion mode and a positive ion mode used in order to view the ions; mass spectrometry is not capable of looking at both the positive

and negatively charged atoms at the same time due to the electric fields needed to move and guide the ions. The sample is introduced to the mass spectrometer through an inlet system which introduces the sample to the ionization source and may take the ions from atmosphere to vacuum $10^{-4} – 10^{-8}$ torr. Vacuum is important in order to have control over the ions, and to reduce collisions with the wall, other ions, and other molecules. If the ions collide, they will be pushed off course and potentially lose their charge, so it is essential to run under very high pressures. The vacuum system consists of mechanical pumps bringing the vacuum to 10^{-3} torr and turbomolecular, diffusion, or cryogenic pumps bringing the vacuum even lower to conserve the ions. The sample is ionized by an ionization source, so that the sample can be analyzed. The gas-phase ions are directed and sorted based on their m/z distribution by a mass analyzer or a series of mass analyzers[2] then a detector imputes the m/z ratio and intensity of the ions to the computer and a mass spectrum is obtained shown in figure 1.1. Some ionization sources fragment the ions and fragment patterns are used to identify the ions along with the parent ion m/z. Other ionization sources do not fragment the ions, but the mass analyzers are capable of trapping the ions and fragmenting them or coupling two systems together in order to trap and fragment ions. Fragment patterns as well as the isotopic distribution and mass to charge ratio are used to identify unknown compounds. Multiple different ionization sources and detectors are available. Depending on the analyte and experiment being performed different systems are preferred. Things to take into account are: ionization of the analyte, separation of multiple analytes, m/z, and run time.

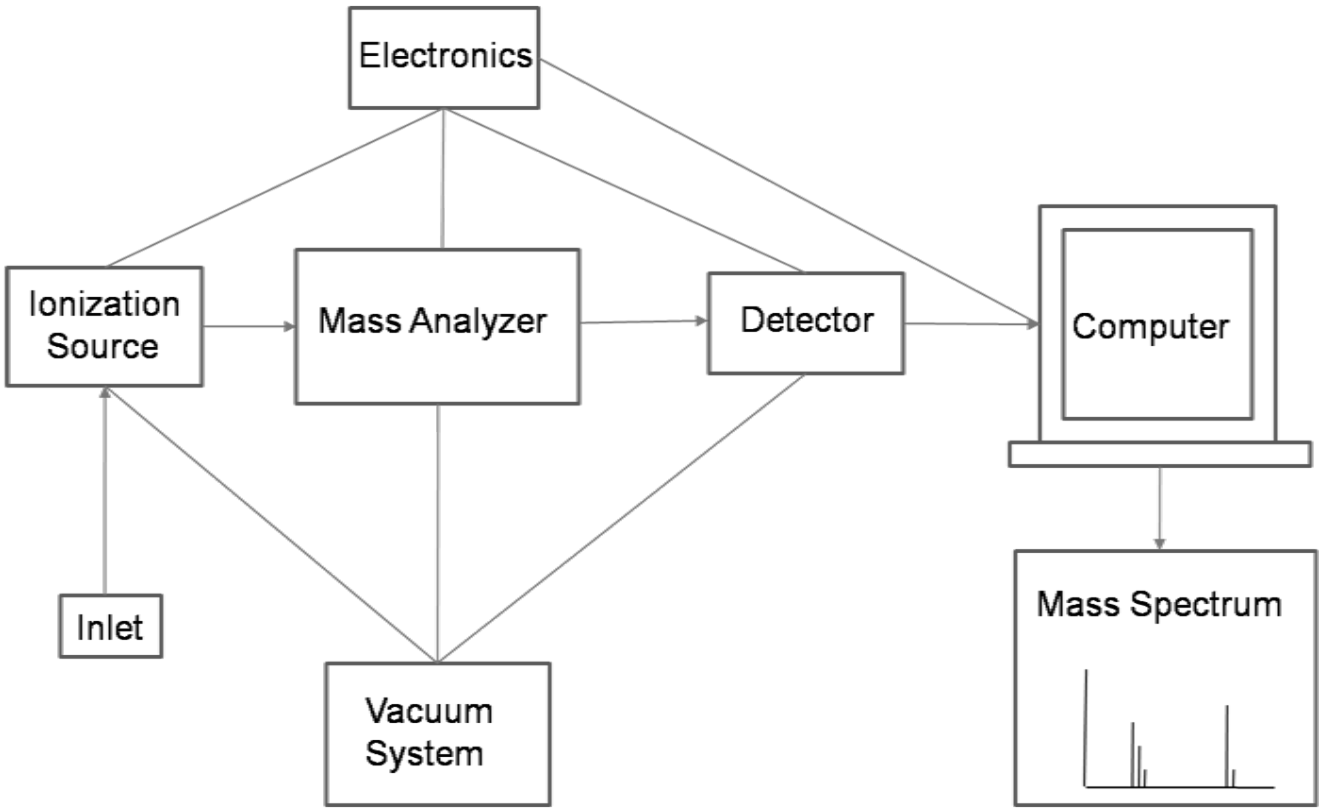


Figure 1.1

Diagram showing the mass spectrometer starting with the inlet and ending with the mass spectrum.

Different mass spectrometry systems have different mass ranges that are analyzed; mass spectrometers can be coupled with other instruments such as liquid chromatography or gas chromatography which separate the sample into individual parts. All these things need to be considered to pick the correct mass spectrometer for the job at hand.

1.2 Introduction to Electrospray Ionization Source

Electrospray is an ionization source that takes solvated charged molecules, and transfers them to gas phase ions, which are then analyzed using mass spectrometry (MS). It is a useful technique, because the desolvation of the analyte occurs slowly making it a very soft process, reducing fragmentation. Generally polar solvents are used, because they form charged droplets easily. The ions are formed through the adduction of a salt or proton with a neutral molecule, reduction and oxidation reactions can also be used to create the ions[3] .

The electrospray setup consists of a metal capillary located 1-3cm away from a counter electrode that has an opening to the mass spectrometer. A voltage of 2-3 kV is applied to the metal capillary which creates an electric field highest at the capillary tip due to its small size. The electric field creates a Taylor cone[4] created by repulsed positively charged ions moving to the meniscus of the cone and negatively charged ions are replaced and push to the middle of the cone shown in figure 1.2. The capillary tip field E_c is calculated using this relationship to form a Taylor Cone.

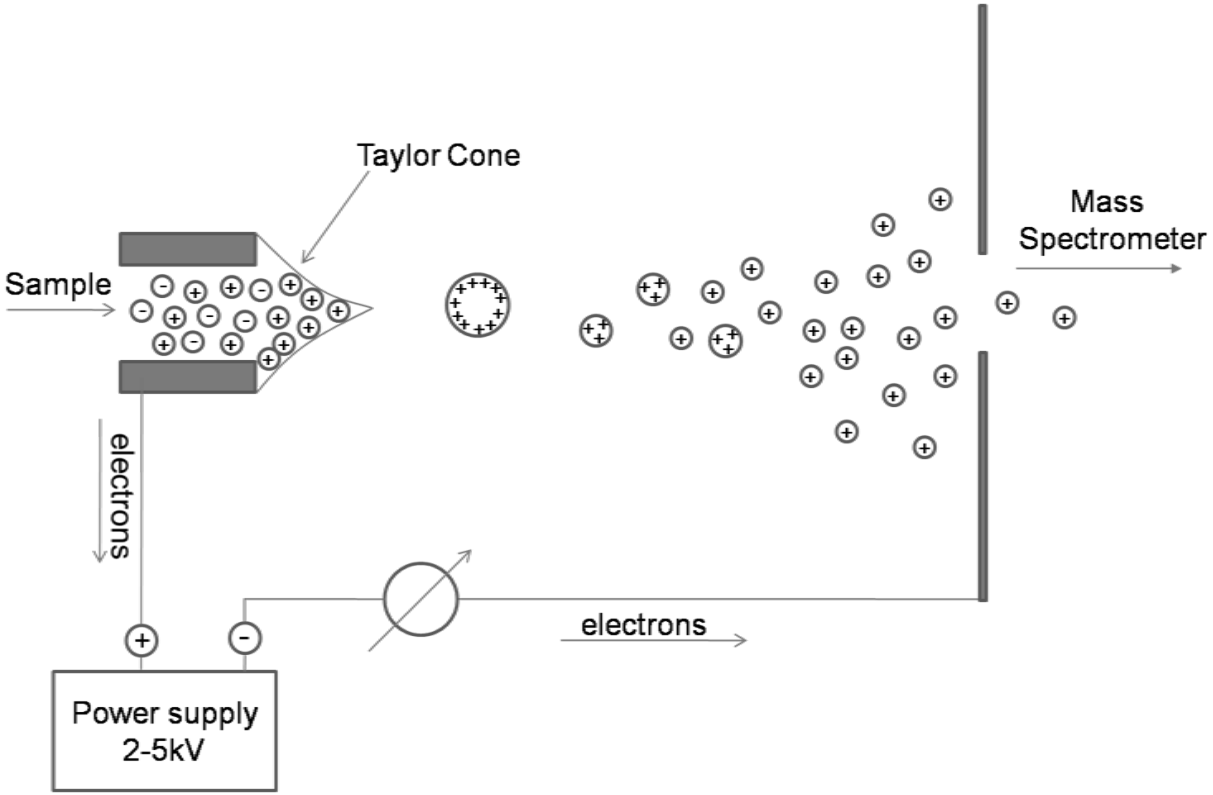


Figure 1.2

The electrospray ionization source showing the Taylor Cone and production of charged droplets.

$$E_c = \frac{2V_c}{r_c \ln\left(\frac{4d}{r_c}\right)} \quad (1)$$

V_c = applied potential

r_c = capillary outer radius

d = distance of capillary tip to counter electrode

The positively charged molecules at the outer edge of the cone expand because they are repulsing each other. The cone then breaks off at the tip forming a charged droplet if the capillary tip electric field is high enough. The droplets are either positively or negatively charged depending on the initial positive or negative voltage applied to the capillary. This is called the electrophoretic mechanism which creates the charged droplets; these equations[5] explain the electric field and potential needed.

$$E_{on} = \left(\frac{2\gamma cos\theta 2}{\epsilon_0 r_c}\right)^{1/2} \quad (2)$$

$$V_{on} = 2 \times 10^5 (\gamma r_c)^{\frac{1}{2}} ln\left(\frac{4d}{r_c}\right) \quad (3)$$

E_{on} = required electric field at capillary tip to induce charged droplet formation

V_{on} = potential needed to induce charged droplets

γ = surface tension of solvent

ϵ_0= permittivity of vacuum

r_c = radius of capillary

θ = half-angle of the Taylor cone

After the droplets are formed they start to shrink due to solvent evaporation which increases the droplets charge. When the ions repulsion with each other is high enough to overcome the surface tension called the Rayleigh stability limit[6] the droplet undergoes coulombic fission increasing the surface area initially stabilizing the smaller charged droplets until the cycle takes place again.

$$q_{Ry} = 8\pi(\epsilon_0 \gamma R^3)^{\frac{1}{2}} \quad (4)$$

q_{Ry} = Rayleigh stability limit

ϵ_0= permittivity of vacuum

γ= surface tension

R= radius

There are two explanations of how gas phase ions are formed. One process is explained by the Iribane and Thomson's theory[7, 8] of ion evaporation where gas phase ions are released from the droplets surface due to the electrostatic repulsion, and the other process is explained by Dole's theory[9] of the coulomb fission mechanism where coulombic fission takes place until only a single gas phase ion is left. Neither mechanism has been proven but both can be used to explain the experimental data.

1.3 Introduction to Nanospray

Nanospray[10-13] ionization is a miniaturized version of electrospray ionization (ESI)[14] ; nanospray ionization has improved total efficiency compared to ESI. Total efficiency is the ions detected divided by the analyte molecules sprayed and is dependent on the desolvation ionization and transfer efficiencies. Nanospray ionization flow rate is reduced compared to ESI; nanospray has a flow rate of 10-40nL/min compared to 5-200ul/min of ESI. The droplet emission size is proportionally related to two thirds power of the flow rate. The nanospray system improves desolvation through forming smaller droplets creating single gas phase ions and eliminating solvent clusters[12] . The average droplet is 200nm allowing for faster evaporation. The transmission frequency is also improved, because the nanospray's Taylor cone is smaller, so the tip can be moved closer to the inlet improving the transmission[12] . The ionization efficiency is also improved through the increase of charge to volume ratio compared to ESI[12] . A gas tight syringe is used to apply pressure to the nanospray tip bringing the sample to the ionization source. A nanospray tip consists of a small capillary with a conductive metal coating; they come open or closed. The open tips have a fixed diameter opening which allows for consistent measurements to be taken and the concentrations to be determined. The nanospray capillaries are only used once eliminating cross contamination between samples that can occur in ESI. Nanospray uses less sample than ESI; a minimum of 300nL is needed, and it has a stable flow allowing for longer running times giving experimentalists more time to analyze dilute compounds and run MS^n experiments. Higher salt concentrations

100mmol/L are tolerated in the nanospray compared to electrospray due to the stabilized flow of ions[12] allowing for less sample preparation.

1.4 Introduction to Quadrupole Ion Traps

Quadrupole ion traps, a mass analyzer, are also called Paul traps[15, 16] ; they consist of 2 hyperbolic end caps and a ring electrode. There is a limited stability region for ions trapped giving the mass range of the trap 2000amu which can be shifted[17, 18] . The Mathieu equation is used to locate the stability region of the quadrupole ion trap.

The generalized Mathieu equation and Mathieu coordinates describing the quadrupole stability region shown in figure 1.3 are given by

$$\frac{\mathrm{d}^2u}{\mathrm{d}\xi^2} + (a_u - 2q_u \cos 2\xi)u = 0 \qquad (5)$$

$$a_u = a_z = -2a_r = \frac{-8zeU}{m\omega^2 r_0^2} \qquad (6)$$

$$q_u = q_z = 2q_r = \frac{4zeV}{m\omega^2 r_0^2} \qquad (7)$$

a_z = Mathieu coordinate

q_z = Mathieu coordinate

$\xi = \omega t/2$

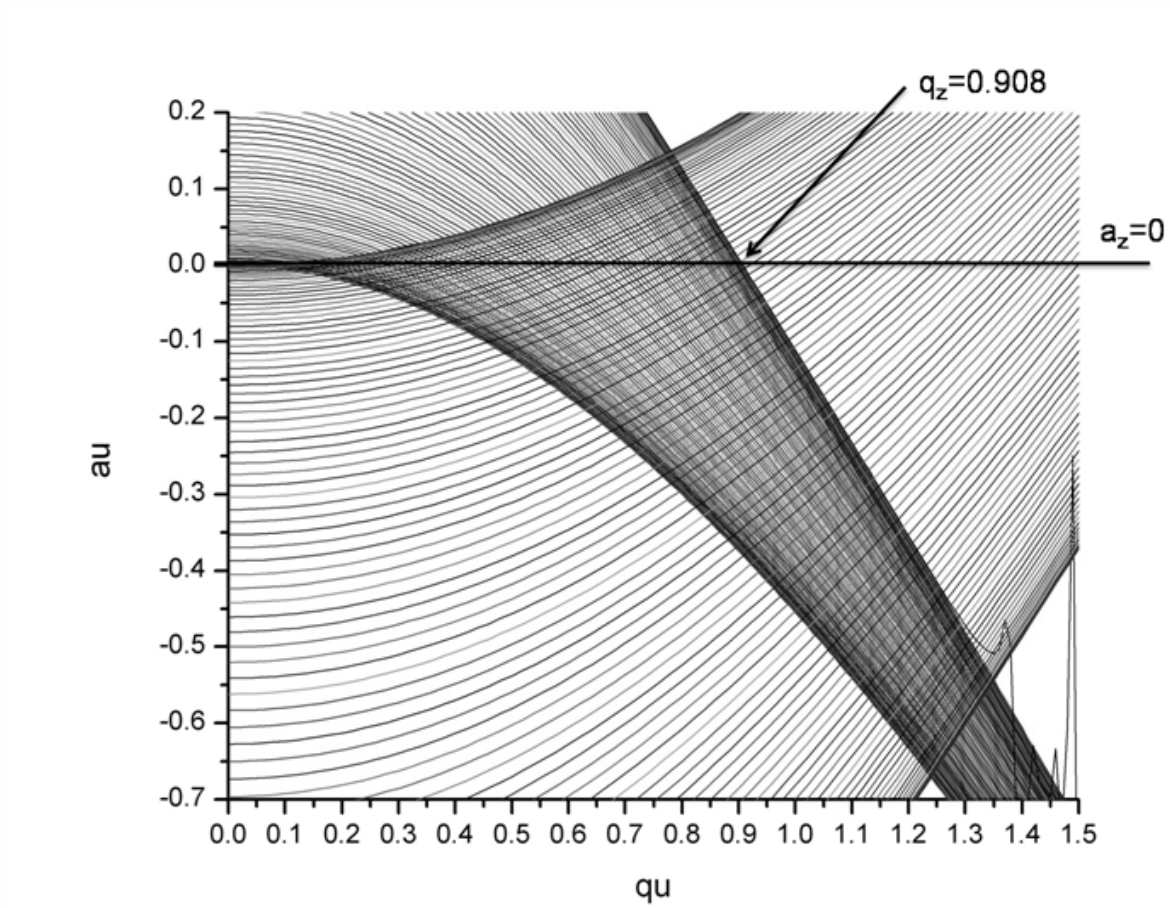


Figure 1. 3

The stability region of the quadrupole ion trap defined by the Mathieu coordinates.

t = time

u = Transverse displacement in x and y direction

ω = angular frequency

U = dc potential

V = rf potential

z = charge

m = mass

Quadrupole ion traps use oscillating electric fields to trap ions in 3-dimentions based on their m/z. The molecules are slowly pushed from the trap by group by changing the applied radio frequency to the ring electrode which destabilizes the ions, and they are ejected based on their m/z[17] . Quadrupole ion traps by mass selecting forward and backward have the ability to trap a single m/z, by exciting the trapped ions, they collide with the neutral gas molecules and fragment. The fragment ions are still in the trap then they can either be analyzed or another fragment trapped and then fragmented MS^{n}[17] .

1.5 Introduction to Microscopy

Microscopes are an invaluable tool in the biological and forensic sciences, because they allow microscopic systems and objects to be visualized. Optical microscopes magnify and resolve images that are as small as 200nm which is the optical limit[19] . Microscopes consist of a light source used to illuminate the image including different types of lamps and lasers. A series of lenses, including condensers and

objectives, are used to focus the light, magnify, and resolve the object; a detector is then used to view the image formed. Resolution and magnification are the two major points of discussion.

Microscopes magnify and resolve images. The magnification is simply increasing the size of the image and the resolution is being able to distinguish between two points. The optical limit is the highest magnification we can achieve for each objective maintaining resolution. If we increase the magnification beyond this point then we achieve empty magnification resulting in an unclear image. Resolution is the ability to distinguish two points and is mathematically defined as

$$\text{Resolution}=\frac{1.22\lambda}{(\text{NA(obj)})+(\text{NA(cond)})} \tag{8}$$

The resolution is the dependent on the numerical aperture NA of both the objective and condenser and λ is the imaging wavelength. It is the goal to match the resolution and magnification to arrive at the best image. Numerical Aperture is the ability to focus the light and resolve a fixed distance away object which is important in resolving the image.

$$\text{NA}=n\sin(\mu) \tag{9}$$

μ = .5 angular aperture of the objective

The total magnification is determined by multiplying the objective lenses magnification by the ocular lenses magnification[20] .

1.6 Fluorescence Microscopy

Fluorescence is the process of electrons being excited to a higher energy state by incoming excitation wavelengths, then the excited electrons fall back to the ground state (lowest energy state) releasing a photon with a longer wavelength and less energy than the incoming excitation wavelength. A series of filters and mirrors are used to focus the wavelength of interest and are used to retrieve the emitted wavelength coming back filtering out the background noise from the rest of the sample.

The first filter only lets the excitation wavelength through; the dichroic mirror bends the light and sends it through the objective to the sample. The fluorescent signal is sent through the dichroic mirror and then is filtered by a barrier filter that filters out any residual excitation wavelengths reflected by the sample and then the fluorescent signal is sent to the detector[19] forming an image.

These microscopes are important in the biological sciences and allow for specific areas of a cell to be stained (attached to fluorophores) and visualized. Only the areas that are stained show up against a black background unless parts of the cells auto-fluoresce. Multiple areas of the cells can be stained with different fluorophores of different excitation wavelengths allowing for multiple filter cubes to be used to see the different fluorophores and then the pictures can be overlaid giving a complete view of all the fluorophores and stained areas of the cell.

CHAPTER 2

THE MULTISTAGE BIOWORKSTATION: DESIGN AND APPLICATIONS

2.1 Abstract

The multistage bioworkstation is wide-ranging in its applications in the biological, chemical, and forensic sciences. The bioworkstation consists of a platform with four nano-positioners that hold end-effectors and capillaries used to manipulate, probe, and characterize objects of interest. The multistage bioworkstation has been coupled to nanospray mass spectrometry allowing for precise and accurate analysis of cellular and trace analytes. We demonstrate this technique by probing caffeine and histidine from individual fibers and analyzing them using nanospray mass spectrometry. The technique is also demonstrated through the indirect probing of lipid bodies from cotton seeds. Using a single instrument to manipulate, probe, and characterize an analyte minimizes the need of having multiple devices and instruments; this saves time moving devices and instruments on and off the microscope stage through different parts of an experiment. There are future forensic and biological applications of the multistage bioworkstation including gunshot residue analysis, toxicological studies at the cellular level, and liquid-liquid microphase extraction.

2.2 Introduction

There are many types of microscopes and mechanical devices to choose from when performing cellular manipulation. Inverted microscopes are preferred when using a

micromanipulator or an optical trapping system, because they allow room for the manipulator needed to probe a system. Confocal microscopes are also used, because they allow for larger working distances of 10-50um vs. 2um of wide-field systems allowing multiple layers of a tissue and cell to be in focus. Mechanical devices include micromanipulators, optical traps, and microelectromechanical systems (MEMS)[21] that are used in order to isolate and manipulate cells. Microfluidic systems are used for separating cells, electrophoresis and performing bioassays on a single chip[22] . Microfluidic systems have been directly infused into ESI-MS and now systems have also achieved nano-flow rates[23] . Optical trapping is being used to manipulate cells as well as bacteria[24] . It uses a laser to trap nano-particles or cells[25] , also micromanipulation is being used to sort and isolate cells. MEMS are also being used to isolate cells and analyze them[21] . The multistage bioworkstation incorporates all of the needed operations into one unit by the simple exchange of an end-effector eliminating the need for multiple devices.

Micromanipulation is a significant tool in the biological and chemical sciences, being used to manipulate nano-particles and cells, because of its precise motions. In the biological sciences, it is currently being used to transfer single cells [26] , and to isolate specific bacterial cells from a group[27] , and it has also been employed for sample preparation on MS analysis[28] . Micromanipulation has also been used to extract mitochondria from cells which were then analyzed using electrophoresis [29] . Nanomanipulator's resolution is increased compared to micromanipulators in lieu of the optical limit, allowing for precise movements minimizing cell damage and therefore advances can be made in the biological sciences.

Trace analyte analysis is made possible by using highly sensitive instruments including mass spectrometers that are mass accurate and specific. Nanospray[11, 12, 13,] is miniaturized version of electrospray ionization [14] and is not as sensitive to high salt concentrations as electrospray minimizing sample preparation [30] . Nanospray has a low flow of 20-50nL/min, so only a minimum of 300nL volume is required and 300attograms of analyte is needed to analyze an analyte making it an ideal ionization source to couple to the bioworkstation. LC-ESI-MS has been used to analyze trace explosives [31] . Nanospray mass spectrometry is a useful in trace analyte analysis as well as cellular analysis due to its reduced sample sizes and reduced sample preparation.

2.3 Results and Discussion

2.3.1 Instrumentation

The multistage bioworkstation is a commercial nanomanipulator stage (L200, Zyvex, Richardson, TX) coupled to (LCQ Deca XP Plus, Thermo Fisher, San Jose, CA) with a (Nanospray Ionization Scource, Proxeon Biosystems; Odense, Denmark) to collect the mass spectra of the ions. The multistage bioworkstation is mounted to (TE2000U Microscope, Nikon; Melville, NJ) and the nano-positioners are attached to (PE2000b 4-Channel Pressure Injector, MicroDataInstrument Inc.; S. Plainfield, NJ).

2.3.2 General Design of the Multistage Bioworkstation

The multistage bioworkstation consists of a nanomanipulator stage with four nano-positioners which are attached to a cabinet with a piezo voltage source and a

pressure injector shown in figure 2.1. The bioworkstation is mounted to the stage of the inverted microscope; it can be easily transferred to other microscopes enabling us to have access to different types of visualization confocal vs. wide-field. It can hold up to eight nano-positioners allowing multiple probing tips and end-effectors to be used if needed. There are two coarse mode nano-positioners which are driven by a stick slip drive. It has a PWM signal that is applied to an arm with a ceramic bead attached to the end. The arms path is an oval; the band sticks to a ceramic plate then slips back which repeats the cycle to generate the movement. The range of motion is 12mm in the X and Z axes and 28mm in the Y axis with a resolution of 100nm. There are two fine nano-positioners have coarse mode abilities as well as an optional fine mode that has a 10nm resolution. The fine mode nano-positioners are driven by a piezo-electric crystal, when high voltage is applied to the crystal it expands. The geometry and orientation of the crystal is responsible for the range of motion in each axis (X,Y,Z). The range of motion of the fine mode is 100um in the Z and X axes and 10um in the Y axis. We have an added motion in being able to manually tilt the nano-positioners in total we have 4 degrees of motion.

The nano-positioners are controlled by a joystick and computer program that can be made to automate the nanomanipulator with functions of auto-lift, lifting all the positioners to the maximum height in the fine mode, and other scripts can be written.

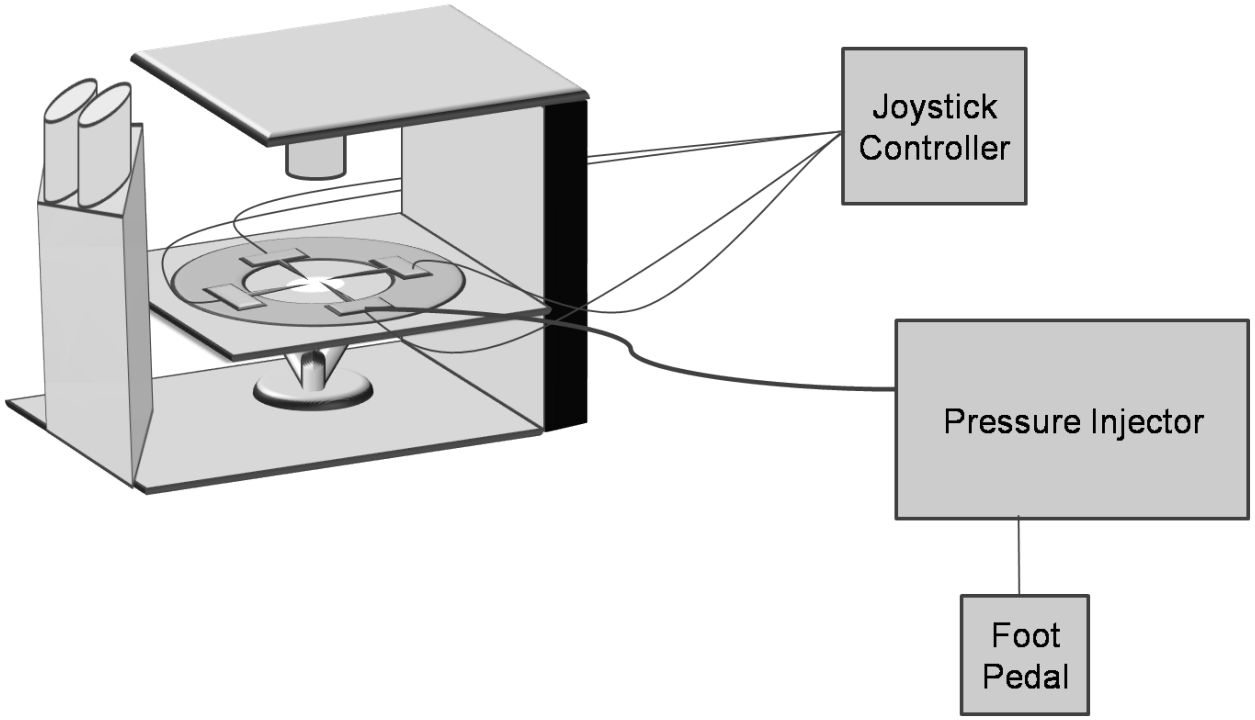


Figure 2. 1

The diagram of the multistage bioworkstation mounted to an inverted microscope controlled through a joystick and connected to a pressure injector.

2.3.3 Coupling the Multistage Bioworkstation to Nanospray Mass Spectrometry

The bio-workstation has been coupled to nanospray mass spectrometry shown in figure 2.2. This coupling was made possible by adjusting the coarse nano-positioner to hold a nanospray tip. The process includes filling a nanospray tip with a nanospray solvent and an adducting ion then inserting it into the nanospray source and breaking the tip open. The nanospray tip is then transferred to the nano-positioner and the tip landed near the object of interest. The pressure injector gives a controlled amount of pressure to the tip to inject the solvent and dissolves the analyte of interest then pulling the dissolved analyte back into the tip. The nanospray tip is then transferred to the nanospray source and the analyte is analyzed based on its m/z as well as its fragment pattern, MS^n, this coupling allows for the analysis of multiple compounds.

The bio-workstation can also be coupled to other instruments including microfluidic systems allowing for the separation and analysis of a mixture of compounds. Microfluidic systems are now being directly coupled to mass spectrometry to analyze samples allowing for future sampling, separation, and analysis.

2.3.4 Positioners and End-Effectors

The capillary tips are attached to the nano-positioners shown in figure 2.3a which are connected to a pressure injector with an injection pressure of 60psi and a fill pressure of 24 mmHg. Capillary tips can be used to sample directly from cells shown in figure 2.3b. We are capable of setting up sequences with up to four capillaries in order to increase our productivity and expand the types of experiments that can be performed.

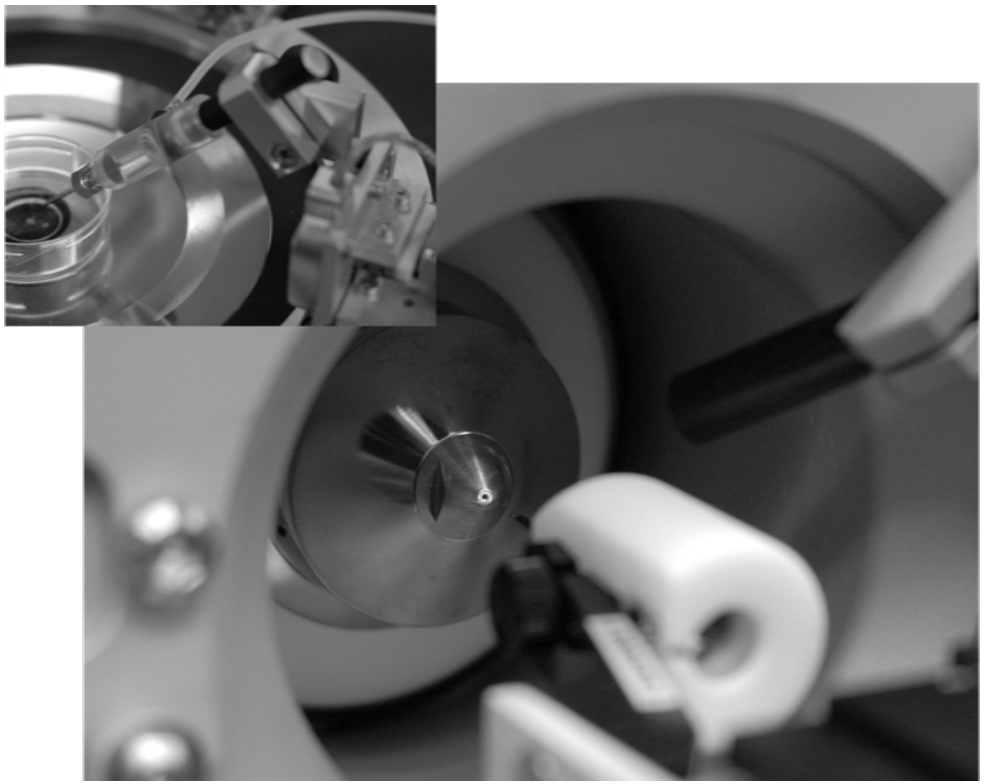
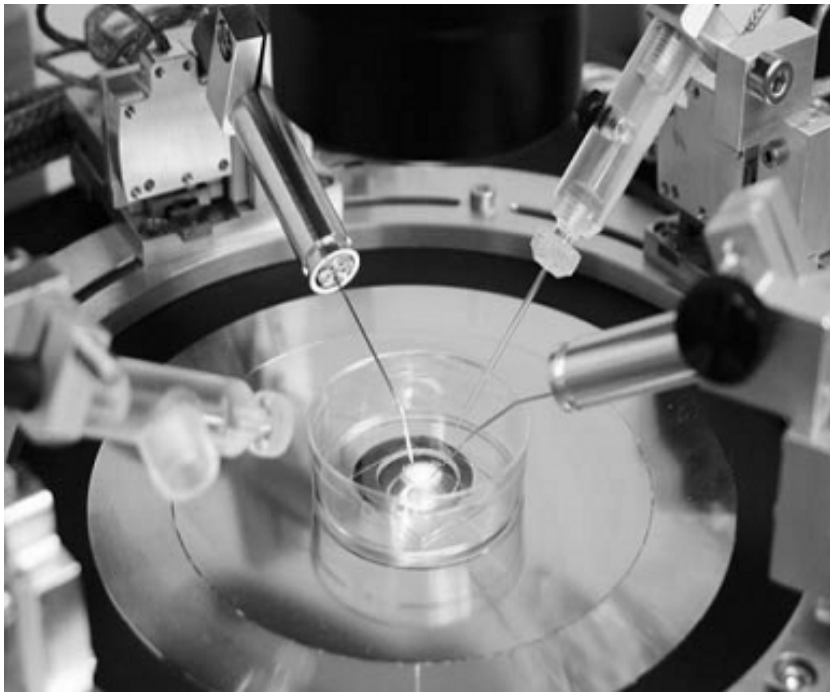


Figure 2.2

Figure showing the coupling of the multistage bioworkstation with nanospray mass spectrometry. (a) Shows the nanospray tip in the nano-positioner probing analyte. (b) Shows the nanospray source head with the nanospray tip transferred from the nano-positioner.



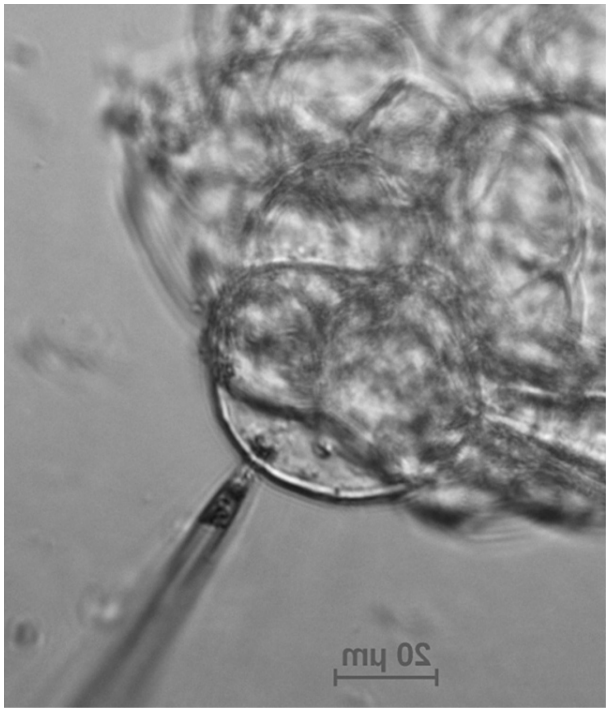


Figure 2.3

Figures a and b show the end-effectors (a) The nanomanipulator with two tungsten probes and a capillary attached to the nano-positioners. (b) The 0.2um capillary probing a *Arabidopsis thaliana* cell.

The pressure injector has a hold function allowing a beveled capillary tip to hold a cell if necessary. Capillary tips as small as 1um with using a pressure of 60psi to overcome the surface tension of water. It takes less to overcome the surface tension of different solvents with lower viscosity. Surfactants may be used to lower the surface tension and allow the injection and fill to take place.

Electro/mechanical probing tips are important to physically manipulate objects as well as to obtain electrical characterization of cells and nanomaterials. The tungsten probes are shown in figure 2.4 a probing a cluster of *Arabidopsis thaliana* cells. Electrochemical etching was used to etch 1um O.D. tungsten probe tips. A 1M KOH solution in a bridged two chamber glass container was used along with a platinum electrode that was placed in one of the chambers with 12 volts applied and the tungsten metal rod was placed in the other chamber. The tungsten rod is slowly raised out of the KOH using a motor, moving at a rate of 65um/min in order to have a tapered tip with a 1um O.D. The current generally started at 16.5mA and then slowly decreased to near zero, 0.2mA, when the probe tip is electrochemically etched. We observe the tip under the 40x objective to determine the tip diameter and the tip making process to asses if the tip is evenly tapered. It takes less to overcome the surface tension of different solvents with lower viscosity. Surfactants may be used to lower the surface tension and allow the injection and fill to take place.

Electro/mechanical probing tips are important to physically manipulate objects as well as to obtain electrical characterization of cells and nanomaterials. The tungsten probes are shown in figure 2.4(a) a probing a cluster of *Arabidopsis thaliana* cells.

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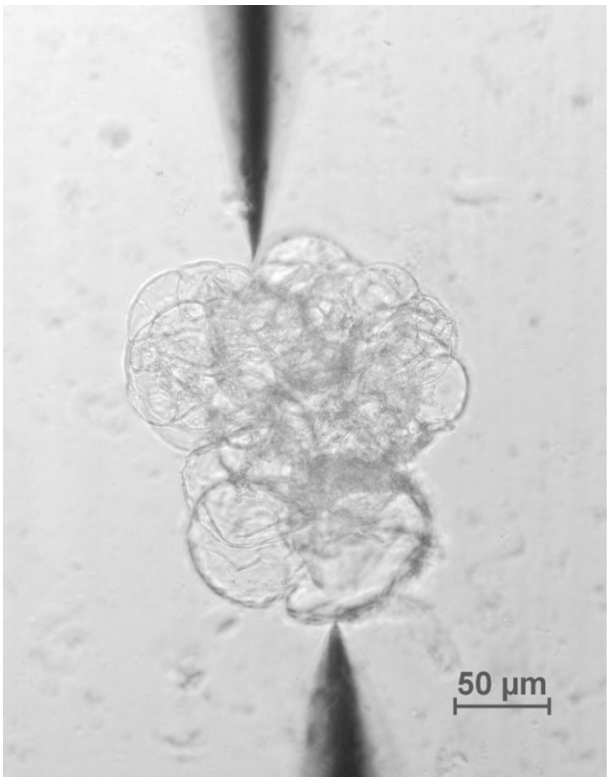
Microgrippers have been designed and made for this bioworkstation, shown in figure 2.4(b). The polymer microgripper is an electrothermal activated gripper using a bent-beam design consisting of SU-8 polymer as the structural material with an electroplated gold heating element. The microgrippers are approximately 60 μm in thickness with a 2 μm Au heating element. They can then be installed as manipulator end-effectors. As end-effectors the microgrippers allow precise, controlled manipulation by varying the voltage applied to grippers. The microgrippers start out in the relaxed, partially closed position. When a voltage is applied the microgrippers expand allowing the microgrippers to be positioned to enclose an object. The voltage can then be decreased to gently grab an object within the opening. Microgrippers can be manufactured with the ability to hold objects of different sizes allowing us to hold a cell in place or a single lipid body. The polymer microgripper's gold heating element is fabricated using typical photolithography techniques, after fabrication the microgrippers are released from the substrate and mounted on a ceramic pad using conductive epoxy.



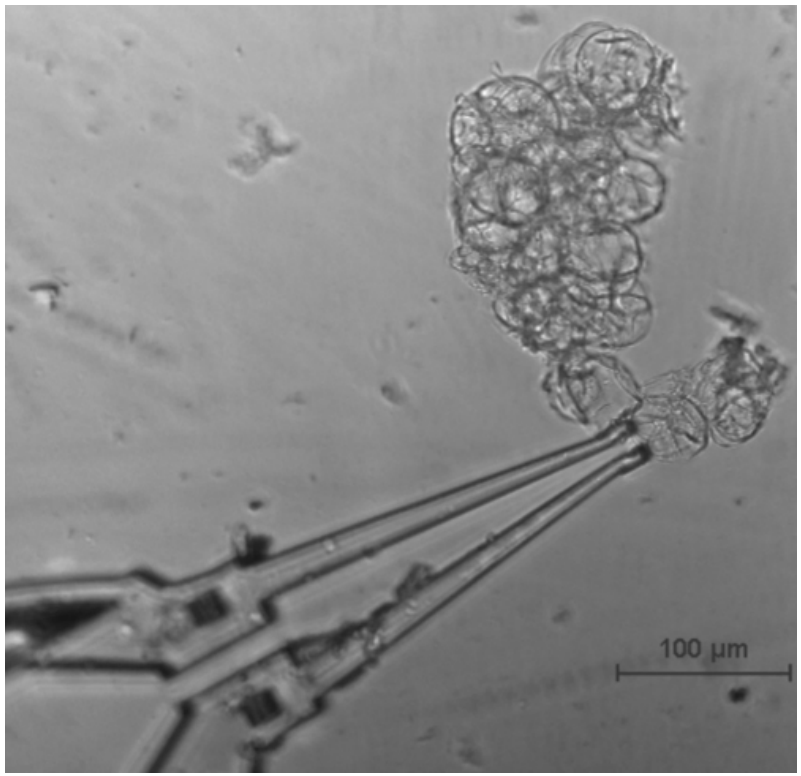


Figure 2. 4
Figures a and b show end-effectors. (a) The tungsten probes near *Arabidopsis thaliana* cells. (b) The microgrippers landing near *Arabidopsis thaliana* cells.

Many other end-effectors are in the process of being designed including a laser that will be able to cut into a cell. The optical trapping may prove to be a useful addition to the multistage bioworkstation as well as other types of probes used to characterize and analyze the different samples and cells including a micro pH meter for the ability to monitor the pH of a single cell. End-effectors can be designed and made to meet many requirements of cellular probing and characterization allowing for an in depth characterization of a single cell.

2.3.5 Overcoming the Optical Limit Using Capacitance

The optical limit is 200nm due to diffraction. The nano-positioners move in 100nm increments and can move 10nm increments in the fine-mode. The inverted microscope is unable to visualize this small of a step; through using capacitance the optical limit will be overcome. This future project will be carried out by using two tungsten probe tips and measuring the capacitance between them, as they get closer together the capacitance goes down. The 10nm movement cannot be visualized using the microscope, but using the tungsten probes the change is capacitance will be seen and the optical limit overcome.

2.4 Applications

Our applications include a variety of cross departmental applications, including direct cell probing and trace fiber analysis. We have demonstrated two useful applications of the bioworkstation coupled to nanospray mass spectrometry: probing trace

analytes from fibers and extracting lipid bodies from cells see chapter 2 and the future work for experimental details. These experiments show some of the many applications of the bio-workstation in an interdepartmental setting of biological, chemical, and forensic sciences.

2.5 Conclusion

The future applications of the multistage bioworkstation are endless. Its probing abilities make is a useful tool for any biologist or forensic scientist. The ability to couple it to nanospray mass spectrometry allows trace analytes to be analyzed. This instrument can be used in a multitude of experiments by switching the end-effectors. New end-effectors are in the process of being made to allow for easier sampling a wide range of objects including cells and trace analytes. The multistage bioworkstation consists of a platform with four nano-positioners capable of holding capillaries and end-effectors. This is an instrument that combines all of the needed elements for biological cellular experiments in one instrument. It has the ability to isolate and hold cells, to probe directly from a single cell, to characterize biological materials. It also has applications in the chemistry and forensic science to characterize nano-particles and to probe trace evidence form single fibers and other surfaces. Future research includes liquid-liquid phase microextraction of organic compounds, more detailed analysis of lipid bodies from cotton seeds, gunshot residue from fibers.

CHAPTER 3

NANOMANIPULATION-COUPLED NANOSPRAY MASS SPECTROMETRY APPLIED TO THE ANALYSIS OF TRACE FIBERS

3.1 Abstract

This paper presents novel instrumentation of nanomanipulation coupled to nanospray mass spectrometry, which is used to directly probe trace analytes from individual fibers. New techniques are needed in order to more accurately probe trace analytes from individual fibers with analyte sensitivity and minimally evasive extraction of the sample. We are presenting new instrumentation, that has direct application to fiber analysis and trace species analysis with the capability to improve the current methods of probing. Our nanomanipulator is capable of 10nm resolution making it ideal for direct sampling of trace analytes, in lieu of the optical limit. This novel technique demonstrates the coupling of the nanomanipulator to nanospray mass spectrometry utilizing tips filled with solvent of interest to retrieve the trace analyte, and then transferring it directly to the mass spectrometer for analysis. This method requires minimal sample preparation, taking the sample straight from the source to the instrument. We will demonstrate the capability of extracting a single particle of histidine or caffeine from an individual fiber, and analyzing directly with nanospray MS. Nanospray MS uses small sample volumes and sample sizes, 300nl volume and 300attogram making it the ideal choice to couple with the nanomanipulator. Future work will be done to show direct cell applications and

trace forensic applications such as gunshot residue analysis and liquid-liquid microphase extraction experiments.

3.2 Introduction

Direct probing from a sample surface directly coupled to mass spectrometry is a useful tool, helping to eliminate sample preparation and analysis time. Currently there are three techniques at the fore front of direct-coupled surface sampling mass spectrometry (MS); desorption electrospray ionization (DESI)[32] , surface sampling probe electrospray ionization[33] , and dielectric barrier discharge ionization source (DBDI)[34] . DESI sprays charged solvent droplets onto an ambient surface which ionizes neutral analytes, which are then desorbed from the surface and analyzed using MS[32 35] . This technique has been used to detect trace amounts of explosives as well as sampling directly from human skin[36 37] . Surface sampling probe electrospray MS uses a liquid junction between the electrospray source and the surface to dissolve and then ionize the analyte, which is then electrosprayed into the MS[33 38] . This method has been used to directly sample drugs from thin tissue slices[39] . DBDI uses a dielectric barrier discharge to create a stable flow of plasma that desorbs and ionizes the sample off of an ambient surface then analyzes it using MS[34] . All of these techniques have great utility, but need a relatively large area, 20-100um, for analysis.

There are currently no methods to probe trace analytes directly from a single fiber. One of the current methods of probing trace analytes is the swab method. An object's surface is swabbed using a textile sampling swab that is then put into solution to

extract the analyte of interest[40] . This method is not the best to collect trace analytes, due to analyte losses and dilution of analyte concentration. Multiple steps, comes with a multiple handling of the analyte, which can lead to sample contamination[41] . Improvement in trace analyte sampling is needed in order to be able to more accurately solve problems and collect trace evidence to increase analyte concentration if there is not enough analyte on a single fiber utilizing the swab method.

Mass spectrometry (MS) is a useful tool, for trace analysis due to its sensitivity, specificity, and mass accuracy enabling the analysis of many different types of compounds.

3.3 Materials and Methods

The solvents and chemicals utilized were Glacial Acetic Acid, Optima* LC/MS Methanol, L-Histidine, and Caffeine (Thermo Fisher Scientific Inc.; Waltham, MA); no further purification was necessary. Millipore water was obtained using the Milli-Q^{UF}Plus (Millipore; Billerica, MA) with better than 18M salt content. We used glass bottom dishes (0) to analyze our samples (Mat Tek Corp.; Ashland, MA), and we probed our analyte from 100% rayon white bemberg lining. We used the following instrumentation: LCQ DECA XP Plus (Thermo Finnigan; San Hose, CA) with a nanospray ionization source (Proxeon Biosystems; Odense, Denmark) was used to analyze the samples. L200 nanomanipulator (Zyvex; Richardson, TX), the TE2000U Microscope (Nikon; Melville, NJ) and the PE2000b 4-Channel Pressure injector (MicroData Instrument Inc.; S. Plainfield, NJ) were used to retrieve the analyte from the fiber.

The Au/Pd plated nanospray tips are loaded with the solvent of interest, and then the tip is broken using the nanospray source head. A blank is run to determine any solvent contamination, and a background spectrum of the solvent is taken. The tip is then transferred to the nanomanipulator for trace analyte probing from a rayon fiber that was doped with the analyte of interest, and placed in a glass bottom dish. The rayon fiber was tacked down to ensure minimal movement of the fiber and analyte on the fiber. The particle of interest was found on the fiber, and then the nanospray tip was landed near it, less than a micrometer away. This process was very tedious and time consuming, the fiber was constantly disturbed by air currents from the air conditioner and vibrations of the table. It only took a slight movement of the fiber to lose the position of the nanospray tip with the analyte and then to have to land the nanospray tip over again near the analyte. Tacking down the fiber helped to eliminate the problem, but it was still a slight issue. We have now inserted the nanomanipulator into a clean room which should eliminate air currents around the sample in the future. The nanospray tip injects the solvent of interest onto the analyte, which dissolves in the solvent. The pressure injector also introduces problems; there are no equations available representing amount of solvent injected based on the pressure, time, surface tension, and inner diameter of a capillary tip. We were unable to quantitate the amount of liquid injecting onto the sample or how much solvent was filled into the nanospray tip. Since every nanospray tip broke at a different diameter we were forced to start with a low injection pressure and slowly raise it up; this did not work very well. It seemed that the pressure increases of 1psi was to high of an increment step, generally when it reached a pressure to overcome the surface tension it would inject

more solvent than desired, and the only way to control it was to use the fill action to balance the back pressure on the capillary tip. The solvent with the dissolved analyte is then retrieved back into the nanospray tip. The nanospray tip is then transferred directly to the nanospray ionization source and the sample analyzed.

Caffeine and histidine are used to illustrate this technique. When sampling caffeine, methanol with 1% glacial acetic acid was used as the solvent, and 3ul of the solvent was loaded into the tip. The tip was landed next to the analyte is shown in figure 3.1(a). The nanomanipulator used an injection pressure of 20.8psi for duration of 11msec delivered from the pressure injector, and a fill pressure of 65psi with a fill time of 50msec. The sample is then analyzed using nanospray MS. When sampling histidine two capillaries were used the nanospray tip filled with 3ul of 60:39:1 MeOH, H2O, and HA the MS solvent and another capillary filled with water seen in figure 3.1(b) The capillary tip injected the water onto the histidine which dissolved in the water and then the nanospray tip retrieved the water with the dissolved analyte. This shows the utility and flexibility of the method. The capillary was injected at 1.3psi for 100msec and the nanospray tip filled at 76.0psi for 0.5 sec repeated once. The sample is then transferred to the nanospray MS and analyzed.

3.4 Results and Discussion

The main purpose of this chapter is to introduce this novel instrument of nanomanipulation coupled to nanospray MS, as an effective tool to analyze trace fibers.

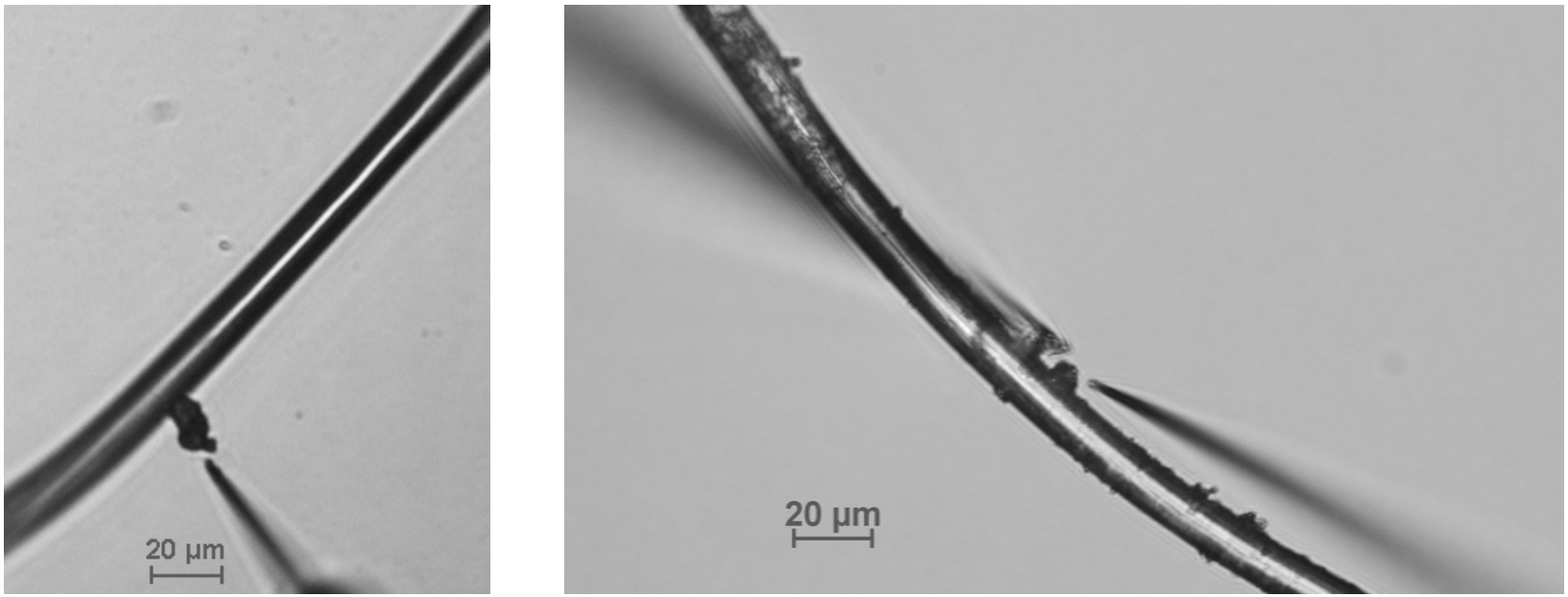


Figure 3.1

Extraction of trace analytes from fibers using the nanomanipulator (a) The nanospray tip landed near the particle of caffeine on a rayon fiber. (b) Histidine on a rayon fiber with a capillary and nanospray tip landed near it.

Both histidine and caffeine trace particles were sampled directly from a single rayon fiber using the nanomanipulator, and then directly analyzed using mass spectrometry.

Figure 3.2 shows the mass spectrum of caffeine (a) and histidine (b) after directly probing trace particles from the rayon fiber. The caffeine particle had an area of 156.65um^2 calculated using the Nikon software to measure the particle. The area of the histidine particle was 47.92um^2 before injection took place. The limit of detection of histidine is 7pg particles when using nanospray, making this an ideal method to analyze and retrieve trace analytes from fibers. Some difficulties with both the histidine and the caffeine were the rate of solvolysis compared to the rate of evaporation of the injected solvent. The small droplets injected onto the particles evaporated fairly quickly not giving the analyte much time to dissolve before the analyte had to be pulled into the nanospray tip. Both the histidine and caffeine dissolved in a high enough concentration that we were able to extract and identify them. Another issue was in the choice of fibers; initially bulk textiles were used consisting of 100 fibers or more. There was difficulty in landing the tip anywhere but the outer edges of the textile. Some of the other fabric choices we chose included cotton which absorbed the injected solvent not allowing the analyte to dissolve or be pulled up. The issue was solved by using a single fiber and switching to rayon textile that did not absorb as much of the solvent. Other issues encountered was the process of breaking the tips open that we saw an signal on the mass spectrometer but the opening was so small we were not able to inject reaching a maximum injection pressure of 60psi with a 1sec hold time. This issue was solved by taking the tip back to the nanospray source and rebreaking the tip.

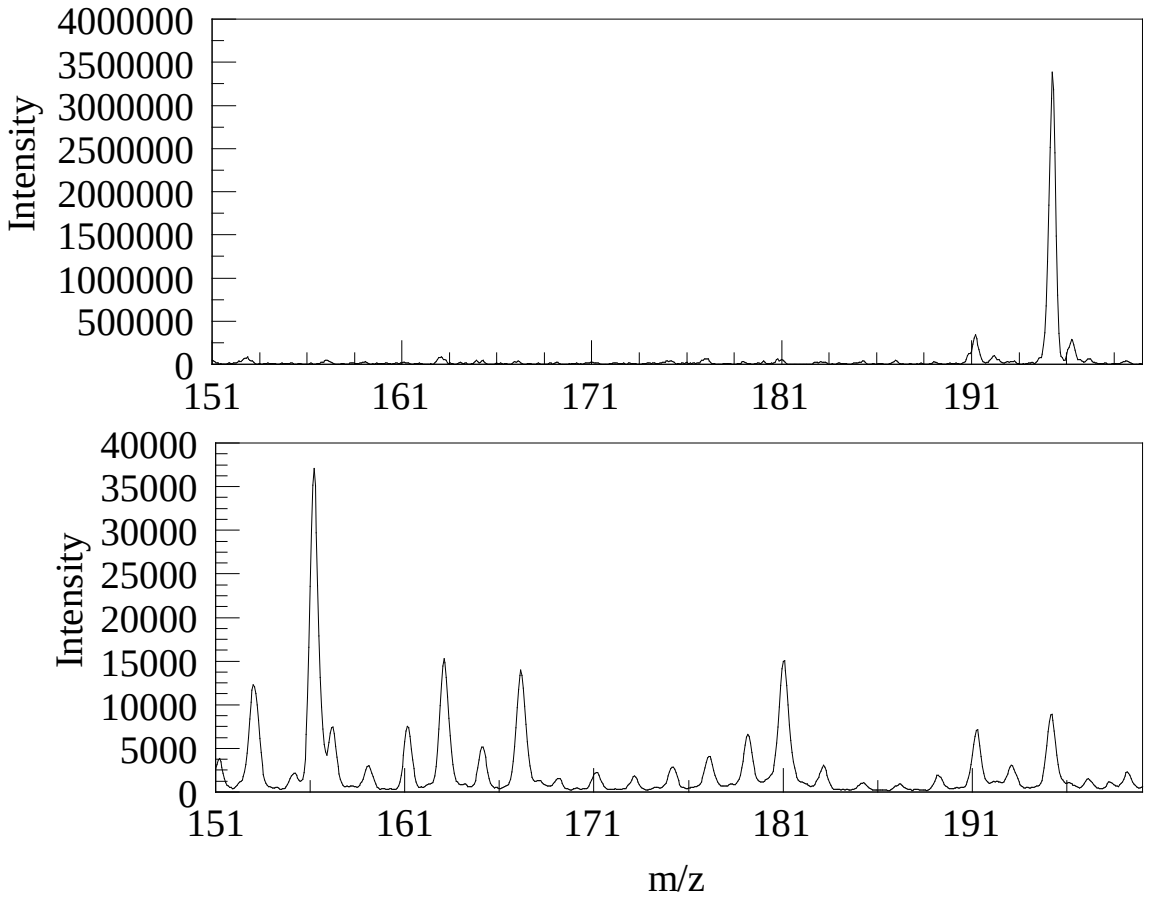


Figure 3.2

The mass spectrum of the trace analytes probed (a) The mass spectrum of caffeine after being probed from the rayon fiber. (b) The mass spectrum of histidine after being probed from the rayon fiber.

Our results clearly show that the nanomanipulator coupled to nanospray MS is an effective instrument to probe trace analytes from fibers. It is an improvement in trace analyte probing from a single fiber, allowing for new experimental procedures to be created, and smaller amounts of analyte to be sampled. The nanomanipulator reduces cost of sampling from fibers, because of the low sample preparation and the reduced sample preparation time and run time. Computer control of the nano-positioners can be created to automate the procedure.

Being able to extract an analyte from a single fiber allows for better analysis of crime scenes, and the reduced sample size and volume required for nanospray MS gives us the ability to retrieve a higher sample concentration. It is important to have a solvent to solubilize the sample as well as give a steady spray flow. Some nonpolar compounds will not dissolve in any of the nanospray solvents, so it is important to utilize a two capillary system with one capillary containing a solvent to solubilize the analyte, and other capillary containing the nanospray friendly solvent that retrieves the dissolved analyte. Some diffusion will occur and the small amount of nonpolar solvent with the analyte of interest will mix with the nanospray solvent, and then can be analyzed using the MS. Another issue is the ionization of the analyte. Multiple different types of compounds can be analyzed ranging from proteins to drugs but if we cannot ionize the analyte we will not see it in the mass spectrometer. Initially bromocresol green was used, but it was not seen in the mass spectrum after having sampled it off of the fiber. The bromocresol green was analyzed at a known concentration of 10ng/ul using ESI. No signal was seen using different adducting ions and solvents; the issue was solved by using histidine instead.

Some analytes will be hard to analyze, because of poor ionization. The correct solvents need to be chosen, and adducting ions need to be chosen, even then some analytes will not be ionized using ESI or nanospray. The inability of knowing what the unknown is can lead to trial and error experiments until the right mixture is found that ionizes the analyte. We are also capable of liquid-liquid microphase extractions to sample trace analytes and gain higher sample concentration from a dilute analyte in a liquid sample and then analyze it. The future work section will illustrate the capabilities of nanomanipulating cells and direct cell sampling.

3.5 Conclusion

Nanomanipulation coupled to nanospray mass spectrometry is a novel technique that we illustrated using caffeine and histidine. We recovered trace particles of histidine and caffeine from different rayon fibers using the nanomanipulator. We were then able to analyze the trace analyte by directly taking the analyte from the source to the nanospray mass spectrometer. This clearly showed the functionality and usefulness of the nanomanipulator coupled to mass spectrometry in the trace analysis of fibers, improving current methods. Future research will include direct cell probing, the retrieval and analysis of gunshot residue from individual fibers, and other trace analysis including liquid-liquid micro-phase extraction.

CHAPTER 4

FUTURE WORK AND CONCLUSIONS

4.1 Cellular Lipid Analysis

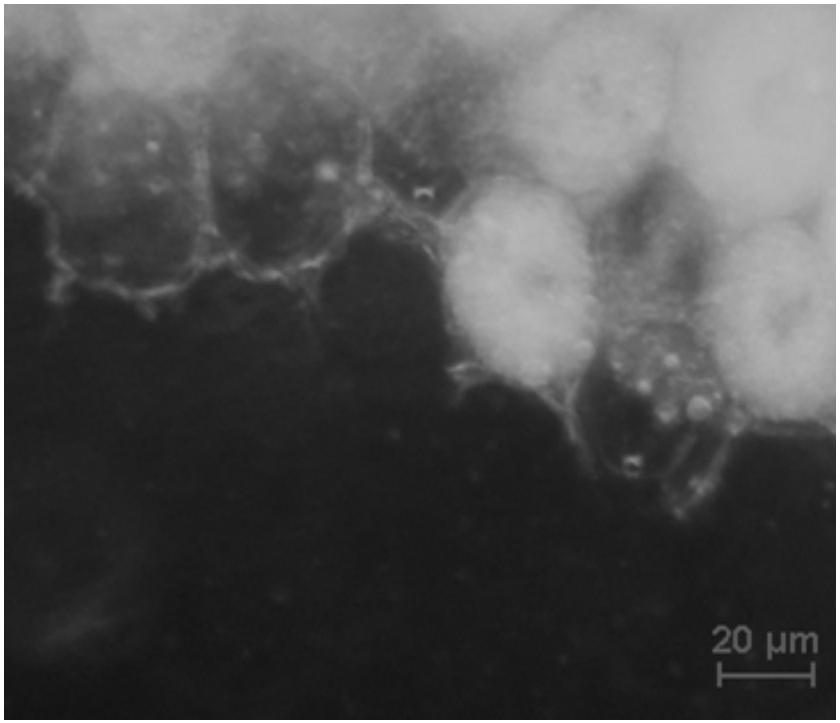
Lipidomics are important on the basis of knowing the chemistry of the cell and the functions of individual cells. The cotton seed cotyledons may have different distribution of lipid bodies throughout the cotyledon. There are vast differences in the lipid bodies between the lipid bodies of the mutant cotton seeds and the native cotton seed. The current technique is to grind down the cotyledons extract the lipids and purify them. This is a multiple step procedure that is time consuming leaving a mixture of all the triglycerides from all of the lipid bodies. Our methods allow individual lipid bodies to be sampled and then the differences between lipid bodies in the same cell and in different tissue can be compared. This allows more to be learned about the lipid bodies organization and function in different tissue locations. The lipid bodies in the cotton seeds are triglycerides with phospholipids in the lipid body membrane. The chains of the triglycerides include linoleic acid which is 60% abundant oleic acid which is 15% abundant and palmitic acid 25% abundant in non-mutant cotton seeds.

Glycerol trioleate was used as a reference peak and for external calibration of the extracted triglycerides. The nanospray tip was easily clogged by the glycerol trioleate a very nonpolar and sticky molecule, because the tips were clogged not much sample was able to get through and the signal was diminished. Rebreaking the unclogged tip became

necessary to regain the lost signal. The glycerol trioleate was used to reach optimal running conditions for the triglycerides. Initially a solvent of 665:225:35 of methanol, chloroform, and 35mM ammonium acetate was used. This did not give any clear results so a higher concentration of chloroform was used 70:30 chloroform:methanol. The chloroform has a low surface tension that the nanospray sprayed at a higher flow rate shortening the analysis time. We had to switch from using 70:30 chloroform:methanol to 50:50 mixture.

It was impossible to ascertain the lipid bodies from the protein bodies and other organelles under the microscope shown in figure 4.1(b), so the lipid bodies were fluorescently stained with 4,4-diflouro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503) purchased from Molecular Probes. The stained lipid bodies then fluoresced bright green making them easily identifiable shown in Figure 4.1(a). The free lipid bodies were diffused into a droplet of chloroform which dissolved them. The dissolved lipid bodies were filled into the nanospray tip containing a solution of 2:3 chloroform:methanol with 10mM ammonium acetate. The nanospray tip was transferred to the nanospray source and the sample analyzed.

Figure 4.2 shows the extracted lipid body mass spectrum of triglycerides extracted from the cotton seeds we see 5 triglycerides adducted to ammonium present in the spectrum $C_{53}H_{102}O_6N$ 848.77 m/z , $C_{55}H_{102}O_6N$ 872.77m/z, $C_{55}H_{104}O_6N$ 874.79 m/z, $C_{55}H_{106}O_6N$ 876.802 m/z, $C_{51}H_{102}O_6N$ 824.77 m/z. We also see peaks at 864.73m/z, 888.80m/z, 890.67 m/z, and 892.67.



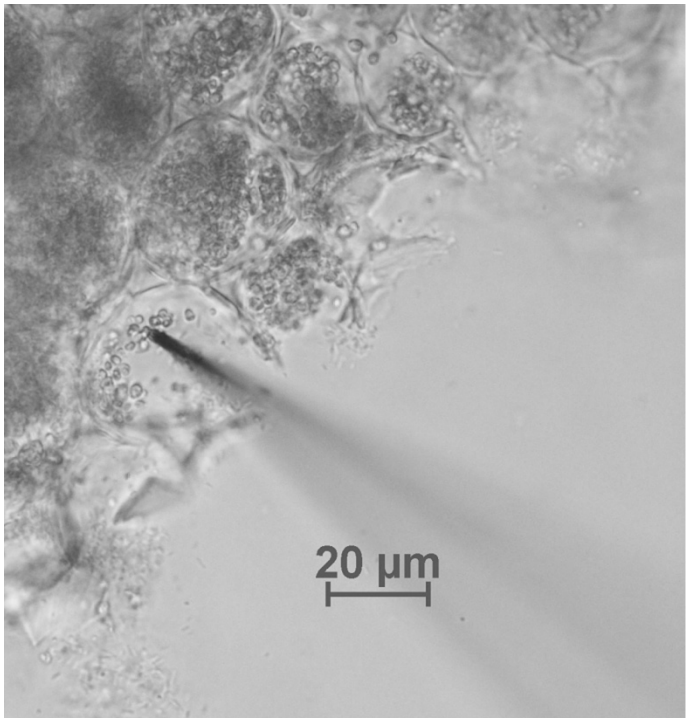


Figure 4.1

The cotton seed cells (a) The fluorescently stained lipid bodies of the cotton seed. (b) A nanospray tip probing a cotton seed cell.

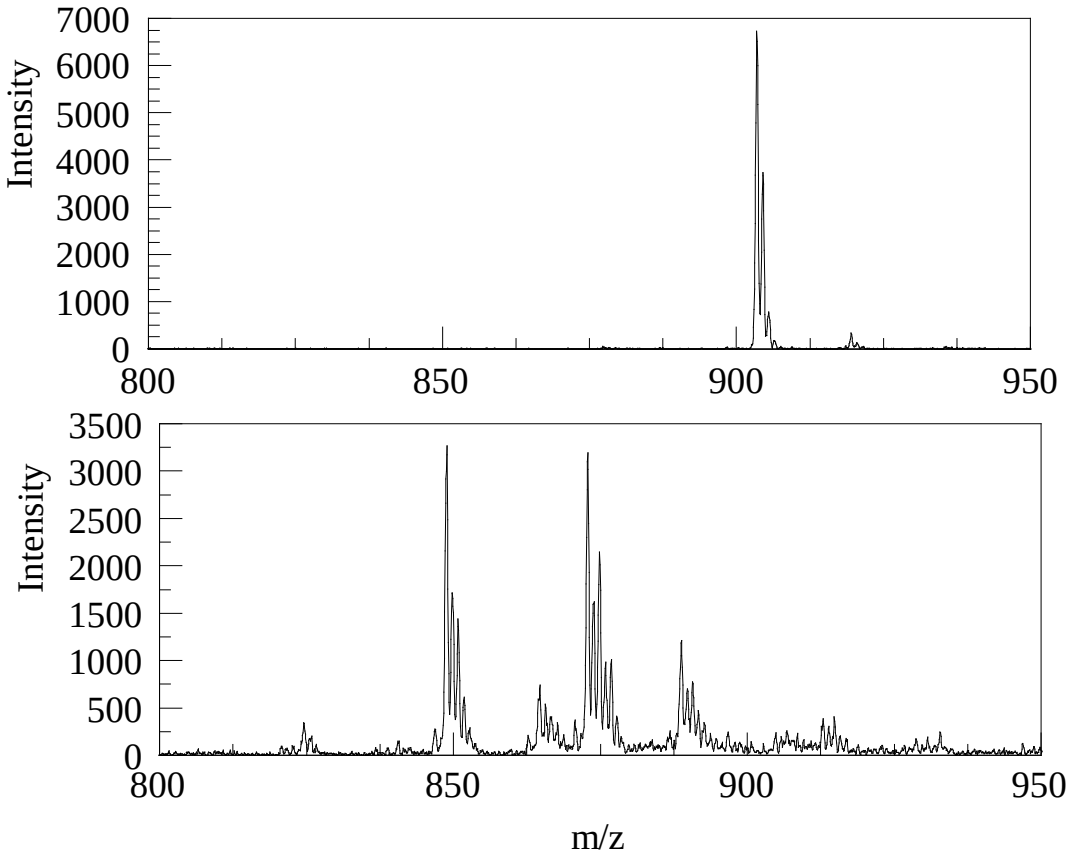


Figure 4.2

The mass spectrum of the triglycerides (a) The mass spectrum of glycerol trioleate used to manually calibrate the instrument. (b) The mass spectrum of the triglycerides extracted from the lipid bodies.

There are all 16 amu apart from their respective peaks the 888.8, 890.67, an 892.67 have the same distribution as 872.87, 874.87, 876.87 they all differ by 16. Glycerol trioleate also has a peak at 918.93, 16 amu apart from the major parent peak. We theorize that oxygen is interacting with a double bond of the parent peak which will be verified in the future using MS/MS. This technique is useful in the biological sciences to analyze individual components of cells. Much can be learned by going to the cellular level and analyzing cells individually.

4.2 Future Work and Conclusion

This summer experiments on gunshot residue will be performed. The residue will be extracted from fibers collected from textiles that have been shot. The gunshot residue particles are nonpolar so chloroform will be used to extract them. Further studies will be performed with the lipid bodies, and they will be extracted directly from cells and analyzed. Also liquid-liquid microphase extraction will be carried out using chloroform to collect organics in water; this method will increase analyte concentration. In the future the multistage bioworkstation will be updated with new end-effectors including lasers, and improved microgrippers. The station will incorporate multiple tools to enable easy access to a number of applications and uses in a single instrument with capabilities to be coupled to multiple instruments including nanospray MS and microfluidic devices.

In conclusion this research has opened the doors in chemistry, biology, and forensic sciences to expand our knowledge of microsystems. This technology has allowed us to probe trace analytes improving the current methods of trace fiber analysis. Cellular

probing has allowed us to analyze lipid bodies without further purification. We will be able to characterize cells and microsamples in order to learn more about them. We will be able to better utilize our resources to probe analytes from cells and surfaces. The utility of this instrument is limitless because new end-effectors can be attached allowing for new capabilities and improvements in the experiments.

REFERENCES

(1) de Hoffmann, E.; Stroobant, V. In *Mass spectrometry :principles and applications;* J. Wiley: Chichester, England; Hoboken, NJ, 2007; , pp 489.

(2) Dass, C. In *Fundamentals of contemporary mass spectrometry;* Wiley-Interscience series on mass spectrometry; Wiley-Interscience: Hoboken, N.J., 2007; , pp 585.

(3) Cole, R. B. In *Electrospray ionization mass spectrometry :fundamentals, instrumentation, and applications;* Wiley: New York, 1997; , pp 577.

(4) Taylor, G. *Proceedings of the Royal Society of London. Series A, Mathematical and Physical Sciences* **1964**, *280*, 383-397.

(5) Smith, D. P. H. *IEEE Trans. Ind. Appl.* **1986**, *IA-22*, 527-535.

(6) Rayleigh, L. *Philosophical Magazine* **1882**, *14*, 184.

(7) Thomson, B. A.; Iribarne, J. V. *J. Chem. Phys.* **1979**, *71*, 4451-4463.

(8) Iribarne, J. V.; Thomson, B. A. *J. Chem. Phys.* **1976**, *64*, 2287-2294.

(9) Dole, M.; Mack, L. L.; Hines, R. L.; Mobley, R. C.; Ferguson, L. D.; Alice, M. B. *J. Chem. Phys.* **1968**, *49*, 2240-2249.

(10) Mann, M.; Wilm, M. *Trends Biochem. Sci.* **1995**, *20*, 219-224.

(11) Wilm, M.; Shevchenko, A.; Houthaeve, T.; Breit, S.; Schweigerer, L.; Fotsis, T.; Mann, M. *Nature (London)* **1996**, *379*, 466-469.

(12) Wilm, M.; Mann, M. *Anal. Chem.* **1996**, *68*, 1-8.

(13) Wilm, M. S.; Mann, M. *Int.J.Mass Spectrom.Ion Processes* **1994**, *136*, 167-180.

(14) Henderson, W.; McIndoe, J. S., Eds.; In *Mass Spectrometry of Inorganic and Organometallic Compounds;* John Wiley & Sons, Ltd: West Sussex, England, 2005; , pp 27

(15) Paul, W.; Steinwedel, H. Z. *Zeitschrift für Naturforschung* **1956**, *104*, 672.

(16) Paul, W.: Steinwedel,Z. Z. *Zeitschrift für Naturforschung* **1953**, *8a*, 448.

(17) March, R. E.; Todd, J. F. J. In *Quadrupole ion trap mass spectrometry;* Chemical analysis; J. Wiley: Hoboken, N.J., 2005; Vol. 165, pp 346.

(18) Dawson, P. H. In *Quadrupole mass spectrometry and its applications;* American Vacuum Society classics; American Institute of Physics: New York, 1995; , pp 349.

(19) Slayter, E. M.; Slayter, H. S. In *Light and electron microscopy;* Cambridge University Press: Cambridge England; New York, 1992; , pp 312.

(20) Murphy, D. B. In *Fundamentals of light microscopy and electronic imaging;* Wiley-Liss: New York, 2001; , pp 368.

(21) Wang, W.; Soper, S. A.; Editors **2007**, 477.

(22) Dittrich, P. S.; Tachikawa, K.; Manz, A. *Anal. Chem.* **2006**, *78*, 3887-3908.

(23) Koster, S.; Verpoorte, E. *Lab Chip* **2007**, *7*, 1394-1412.

(24) Kuyper, C. L.; Chiu, D. T. *Appl. Spectrosc.* **2002**, *56*, 300A.

(25) Neuman, K. C.; Block, S. M. *Rev. Sci. Instrum.* **2004**, *75*, 2787-2809.

(26) Yamamura, S.; Kishi, H.; Tokimitsu, Y.; Kondo, S.; Honda, R.; Rao, S. R.; Omori, M.; Tamiya, E.; Muraguchi, A. *Anal. Chem.* **2005**, *77*, 8050-8056.

(27) Froehlich, J.; Koenig, H. *Soil Biol.* **2006**, *6*, 425-437.

(28) Kajiyama, S.; Harada, K.; Fukusaki, E.; Kobayashi, A. *J Biosci Bioeng* **2006**, *102*, 575-578.

(29) Ahmadzadeh, H.; Johnson, R. D.; Thompson, L.; Arriaga, E. A. *Anal. Chem.* **2004**, *76*, 315-321.

(30) White, T. P.; Dolan, A. R.; Bigwarfe, P. M., Jr.; Higbee, D. J.; Smith, D. R.; Wood, T. D. *Trends Appl.Spectrosc.* **2002**, *4*, 141-154.

(31) Yinon, J. *Adv.Forensic Appl.Mass Spectrom.* **2004**, 231-274.

(32) Cooks, R. G.; Ouyang, Z.; Takats, Z.; Wiseman, J. M. *Science* **2006**, *311*, 1566-1570.

(33) Van Berkel, G. J.; Sanchez, A. D.; Quirke, J. M. E. *Anal. Chem.* **2002**, *74*, 6216-6223.

(34) Na, N.; Zhao, M.; Zhang, S.; Yang, C.; Zhang, X. *J. Am. Soc. Mass Spectrom.* **2007**, *18*, 1859-1862.

(35) Wiseman, J. M.; Laughlin, B. C. *Curr.Sep.Drug Dev.* **2007**, *22*, 11-14.

(36) Takats, Z.; Cotte-Rodriguez, I.; Talaty, N.; Chen, H.; Cooks, R. G. *Chem.Commun.(Cambridge, U.K.)* **2005**, 1950-1952.

(37) Justes, D. R.; Talaty, N.; Cotte-Rodriguez, I.; Cooks, R. G. *Chem.Commun.(Cambridge, U.K.)* **2007**, 2142-2144.

(38) Asano, K. G.; Ford, M. J.; Tomkins, B. A.; Van Berkel, G. J. *Rapid Commun. Mass Spectrom.* **2005**, *19*, 2305-2312.

(39) Van Berkel, G. J.; Kertesz, V.; Koeplinger, K. A.; Vavrek, M.; Kong, A. T. *J. Mass Spectrom.* **2008**, *43*, 500-508.

(40) Bozenbury, R. H.; Debono, R. F.; Danylewych-May, L. L.; Fricano, L.; Kim, L. Patent Application Country: Application: US; Patent Country: US Patent 2005288616, 2005.

(41) Lloyd, J. B. F.; King, R. M. *J. Forensic Sci.* **1990**, *35*, 956-959.