Ambient Ionization: Surface Analysis of Sexual Assault evidence and 2-Dimensional Imaging of Whole-Body Zebra Fish (*Danio rerio*) Using Desorption Electrospray Ionization

Alexander Chramow

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York University
Toronto, Ontario

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Abstract

Desorption electrospray ionization (DESI) is an ambient surface analysis technique capable of producing 2D and 3D images. The ionization mechanism utilizes a pneumatically assisted sprayer to deposit a charged solvent onto a sample surface. Subsequent impacting primary droplets produce ejecting surface secondary droplets containing desolved analytes, which are then detected by a mass analyzer. This thesis explores two fields of application of DESI, forensics and biological tissue analysis. The former involves the analysis of sexual assault evidence, in the form of condoms, lubricants, and their residues as a potential aid in convicting perpetrators. The latter focuses on investigating the potential use of the zebra fish (*Danio rerio*) as a model vertebrate organism for future toxicological and biological research. Whole-body 2D images were created, highlighting areas of interest such as, the brain, spinal cord, liver, and body fat.

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DESI – Desorption electrospray ionization

2D – Two-dimensional

3D – Three-dimensional

MSI – Mass spectrometry imaging

SIMS – Secondary ion mass spectrometry

MALDI – Matrix-assisted laser desorption ionization

LAESI – Laser ablation electrospray ionization

DAPPI – Desorption atmospheric pressure photoionization

PESI – Probe electrospray ionization

IR-LAMICI – Infrared laser ablation metastable-induced chemical ionization

m/z – Mass-to-charge ratio

LDMS – Laser desorption mass spectrometry

nm – Nanometer

ND-YAG – Neodymium-doped yttrium aluminum garnet

Da – Dalton

MS – Mass spectrometry/Mass spectrometer

ESI – Electrospray ionization

kV- Kilovolts

mm - Milimeter

ACN – Acetonitrile

MeOH – Methanol

IPA – Isopropanol

EtOH – Ethanol

PTFE – Polytetrafluoroethylene

H_2O – Water

μm – Micrometer

DMF – N,N-dimethylformamide

THF – Tetrahydrofuran

$CHCl_3$ – Chloroform

pH – Hydrogen ion activity

MS/MS – Tandem mass spectrometry. Mass spectrometric detection of fragmented primary ions

TNT – Trinitrotoluene

RDX – Research Department explosive, cyclonite, 1, 3, 5-trinitroperhydro-1, 3, 5-triazine

HMX – Octogen, Octahydro-1, 3, 5, 7-tetranitro-1, 3, 5, 7-tatrazocine

PETN – Pentaerythritol tetranitrate

C-4 – Composition C-4

Semtex-H – General purpose plastic explosive, composed of RDX and PETN

Detasheet – Flexible rubberized explosive

fg – Femtogram, $1x10^{-15}$grams

pg – Picogram, $1x10^{-12}$grams

DNA – Deoxyribonucleic acid

DART – Direct analysis in real-time

UV – Ultraviolet

cm – Centimeter

ms – Miliseconds

psi – Pound-force per square inch

μL/min – Microliters per minute

WBA – Whole-body autoradiography

LC-MS – Liquid chromatography mass spectrometry

HPLC – High-performance liquid chromatography

CMC – Carboxymethyl cellulose

μm/s – Micrometers per second

CID – Collision induced dissociation

OCT – Optimal cutting temperature (name of an embedding medium)

TBS – Triangle biomedical sciences (name of an embedding medium)

PS – Phosphatidilyserine

PI – Phosphatidylinositol

ST – Sulfatide

LD_{50} – Lethal dose of a substance causing death in 50% of a population

Chapter One – Introduction

Mass spectrometry imaging (MSI) has revolutionized the way scientists analyze and interpret their data. No longer must researchers decipher peaks and graphs to gain knowledge from their experiments. MSI has opened the world to visual data, where we can acquire a plethora of information from a single image. We use our eyes and visual sense a lot to interact with the world and to absorb most of the information we gather from it, from surviving daily life to being bombarded with advertisements and media. It is this comfort and organic attraction to visual stimuli, which has probably helped push the progression and acceptance of MSI as a powerful analytical technique. Since the first conception of imaging in the early 1960's by Castaing and Slodzian, using secondary ion mass spectrometry (SIMS) to analyze inorganic ions from the surface of biological samples,[1] scientists have been pointing their ion sources towards various surfaces from natural to synthetic to solve problems and find answers in a wide range of fields, including: forensics,[2] processing of drugs and metabolites,[3] medicine,[4] lipid characterization,[5] neurochemistry,[6] and the list is growing.

Since the early days of SIMS, there has been a lot of progression in instrumentation of MSI. Most notably with the development of the high-powered matrix-assisted laser desorption ionization (MALDI) in 1985 by Karas and Hillenkamp,[7] and the soft, ambient ionization of desorption electrospray ionization (DESI) in 2004 by R. G. Cooks.[8] In the last decade there have been numerous techniques developed for use in MSI experiments, most of them having slight alterations to already existing ionization principles such as, laser ablation electrospray ionization (LAESI), desorption atmospheric pressure photoionization (DAPPI), probe electrospray ionization (PESI), infrared laser ablation metastable-induced chemical ionization (IR-LAMICI) to name a few,[9] a lot of them favoring the ease of analysis which ambient techniques possess.

The first experiments using SIMS were far simpler in their inquisitions compared to the studies being carried out today. Designed by Herzog and Honig in the 1950's and 60's, its primary use was to analyze metals and oxides.[10] It produced sample ions, for

detection by a mass analyzer, by focusing an ion beam consisting of primary ions (CS^+, Ga^+, Ar^+) to impact a sample surface under vacuum conditions. The cascading energy of the primary ions' impact causes surface bonds to cleave releasing neutrals, molecular ions, and fragmented ions.[10] This approach was appropriate for analyzing inorganic surfaces, however when it came to organic samples, the large amounts of energy transmitted by the primary ions caused excessive fragmentation, damage and low sensitivity.[11] Though, this did not limit the endeavors of researchers to probe at the mysteries of living tissue. Lipid classes attempting to be analyzed were detected by indirect means, by monitoring specific characterizing fragments, such as phosphocholine at *m/z* 184.[12] It wasn't until the conception of cluster ion sources (Au_3^+, Bi_3^+) that organic surfaces could successfully be analyzed and imaged, most importantly intact lipids which can provide crucial diagnostic information. Using clusters, rather than monoionic ions, significantly enhanced the analysis by increasing the amount of secondary ions being ejected from a sample surface, as well as reducing surface damage. This occurred due to the clusters breaking up upon impact with the surface, therefore spreading the energy across.[12]

Although SIMS was pioneering mass spectrometry and surface analysis over 60 years ago and is still in use today, most analyses involving organic material have been taken over by more popular modern techniques such as MALDI and DESI, both having their advantages and limitations. Not to put SIMS in the obsolete category, it is currently the only source which can reach levels of lateral resolution under 1μm and capable of producing ion images of single cells.[13] Although as resolution increases, sensitivity decreases due to a finite amount of target molecules present in such small areas. SIMS continues to demonstrate relevancy in modern research settings. A study released in early 2014 by M. Fitzgerald, et al. describes the use of NanoSIMS with immunohistochemistry to quantify the size, amount, and changes occurring in Ca^{2+} microdomains within neurons affected by trauma.[14] SIMS is a great tool for analyzing elements and small molecules within a surface, however if you want to study proteins, lipids, and other large molecules you'll have to look elsewhere.

Along came the 1990's, and with it came a powerful new technique called MALDI. Although, Karas and Hillenkamp first reported MALDI in 1985, it wasn't until a breakthrough paper submitted in 1997 demonstrating direct analysis on tissue by Caprioli, et al., which caused this new ion source's popularity to grow exponentially.[15] However, before MALDI was what we know it as today, it was called LDMS, or laser desorption mass spectrometry. The ionization principle is similar to that of SIMS, where an energy beam, in this case produced by photons from a laser, impacts a sample surface to cause desorption and ionization. The initial experiments conducted by Hillenkamp and Karas sought to understand the importance of the laser wavelength applied towards sample desorption. They performed these tests on various amino acids and dipeptides.[7] It was discovered that tryptophan possessed the lowest irradiance threshold amongst all amino acids tested, using a 266nm ND-YAG laser. Their experimental results also revealed that tyrosine and phenylalanine had irradiance thresholds that were factors of 2 and 5 larger than tryptophan, respectively, which are concurrent with their order of decreasing classical absorption. It was during these experiments where they noticed that a mixture of tryptophan and alanine could be desorbed at the irradiance threshold of tryptophan, one tenth of that required to desorb pure alanine, producing strong signals for both quasimolecular ions. They discovered that using a strongly absorbing molecule (usually small aromatics) as a matrix and applying a fixed wavelength can aid and enhance ionization of analytes lacking or possessing weak absorbing characteristics. This is accomplished by creation of matrix-analyte co-crystals, which also isolates the analyte and reduces the potential for cluster formation. The matrix establishes a "softer" ionization technique by absorbing the laser energy, sparing the analyte, and causing ionization by proton transfer.[12]

The discovery of the enhancing effects of matrix application enabled the ionization of molecules larger than 1000 Da such as intact proteins and larger peptides, as was first demonstrated in a groundbreaking paper submitted by Caprioli.[15] It was only a few years later that Caprioli's group would release another revolutionary paper describing the imaging of proteins and peptides from mouse brain and human brain tumor xenograph sections.[16] This study demonstrated the importance of protein expression in

differentiating between normal and diseased/cancerous tissue as well as identifying the potential for biomarker discovery for classification of tumors. MALDI-MS has seen great success in the fields of proteomics,[17] lipidomics,[18] and metabolomics,[19] and has great potential for use as a clinical diagnostic tool.[20] Although MALDI is an impressively dynamic technique, it suffers from slight disadvantages. Some inconveniences associated with MALDI are related to operational factors such as ionization occurring within a vacuum, time constraints regarding pretreatment of samples and matrix application, and interfering effects of matrix ions in the low mass to charge (*m/z*) range of the mass spectrum.

Most of the disadvantageous qualities associated with MALDI and previous surface analysis methods no longer represented an issue with a new wave of ambient ionization techniques. The first and most widely known, DESI, was conceived in 2004 by R. G. Cooks and his group.[8] In order to be classified as an ambient ionization technique, a method must meet three requirements. When conducting ambient surface analysis, the sample surface has minimal or no pretreatment prior to ionization. Unlike MALDI, most surface analysis with DESI requires no pretreatment. In some cases washing a sample to remove an impenetrable layer or sample imprinting onto porous surfaces might be beneficial.[21] The second stipulation is that ionization occurs outside the mass spectrometer. The major ionization process in DESI is described by a droplet-pick-up mechanism (figure 1.1).[22] An electrosprayed charged solvent is sprayed onto a sample surface, creating a film in which analytes can be desorbed. Subsequent impacting solvent droplets create ejecting secondary micro-droplets containing dissolved analytes, which then undergo desolvation processes similar to those occurring in traditional ESI (electrospray ionization). And lastly, only ions not the entire sample are introduced into the mass spectrometer for detection. This allows the possibility for successive analysis with complementary techniques depending on the sample's physical integrity as well as instrumental parameters.

Researchers and investigators have determined the possibility of a few mechanisms involved in the ionization process associated with DESI. The first

mechanism to be introduced is widely considered the main mechanism involved with DESI and is chiefly responsible for the ionization of larger molecules such as proteins and peptides. This mechanism is referred to as the "droplet-pick-up", where incoming charged primary solvent droplets impact the sample surface. Surface analytes become solvated and leave the sample surface by means of secondary microdroplets caused by subsequent incoming primary droplets or even within microdroplet jetting from high impacting primary droplets. Ionization finally takes place within the ejected secondary microdroplets, which undergo desolvation processes similar to ESI such as the charge residue model. This model suggests that the droplets undergo evaporation and fission cycles resulting in gas phase analytes endowed with the charge of the droplet.[24] Smaller molecules such as lipids and aromatics are thought to become ionized through a different mechanism, one involving a charge transfer either from a proton or electron. This mechanism doesn't rely on the sample surface being wet by the solvent, such as the previous mechanism. Instead, the charge transfer is commonly occurring between a gas-phase solvent ion and a surface molecule.[23] Ionization of surface analytes can produce either positive or negatively charged ions depending on whether they become protonated or deprotonated. Charge transfer ionization usually occurs through a proton-transfer reaction. The direction of the transfer, either to the solvent or to the analyte, will be determined by the proton affinities of both species, which will also dictate the polarity of the ionized analyte. Analytes that possess a higher proton affinity relative to the solvent chosen will become protonated and therefore become a positively charged ion.

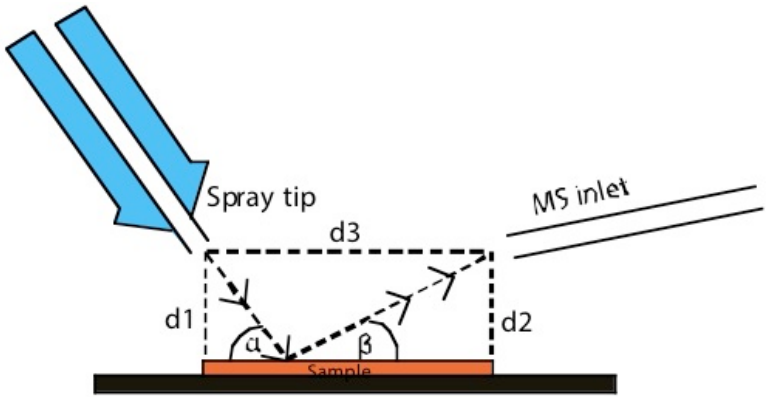

Figure 1.1 - Schematic of DESI Ion source showing operational parameters. Incident angle (α), collection angle (β), spray tip-to-sample distance (d1), MS inlet-to-sample distance (d2), and spray tip-to-inlet distance (d3).

At first glance DESI might seem like a fairly simple technique to pick up, however there are many operational factors influencing the optimization of the instrument. Takáts et al. have previously explored the instrumentation and mechanisms of DESI.[23] Just about every parameter can be tweaked or altered, and in doing so can either yield minor or drastic changes to the ionization efficiency, resolution, signal intensity, as well as causing selective ionization. Some of the major influencing parameters are: spray incident angle (α), spray tip-to-surface distance (d_1), spray tip diameter, nebulizing gas pressure/flow rate, solvent composition, solvent flow rate, magnitude of applied voltage, and orifice diameter of mass spectrometer capillary. The nature of most of these parameters will depend on the characteristics of the analyte. If the analyte being investigated is a protein, peptide, nucleic acid, or carbohydrate it will require different parameter adjustments for optimal signal intensity, compared to smaller molecules such as lipids, aromatics and explosives.[23]

Table 1.1 - Optimal operational parameters for DESI based on analyte characteristics.[23]

Analyte	**Peptides, proteins, carbohydrates, nucleic acids**	**Explosives, lipids, aromatics**
Applied Voltage	1-3 kV	3-8kV
Incident Angle (α)	60-90°	20-50°
Collection Angle (β)	<10°	10-15°
Spray tip-to-sample distance (d_1)	1-2 mm	2-8 mm
MS inlet-to-sample distance (d_2)	1-2 mm	5-8 mm

The optimal parameters for larger molecules, such as proteins and peptides, involves a high incident angle(α), and short spray tip-to-surface distance (d_1). High α angles (60-90°) reduce noise, as well as increase the velocity of the primary droplets thereby increasing the amount of secondary droplets upon impact. Shorter d_1 distances (1-2mm) are necessary for protein ionization since larger molecules require the charged solvent to be present on the sample surface. Higher d_1 values (2-8mm) yield a larger path, which the droplets must travel, giving them a chance to evaporate as well as lose their initial speed. Longer (d_1) distances are beneficial for ionizing smaller molecules, which

do not require charged droplets. Their ionization process occurs through a charge transfer from the gas-phase solvent to the surface analyte, by either proton or electron transfer. This mode of ionization is further enhanced depending on the volatility of the analyte. The magnitude of the applied voltage can also affect the ionization of the analyte. Lower voltages 1-3kV are suited for larger molecules, especially proteins. A low voltage yields a more stable outcome of multiple charge states. Higher voltages tend to yield an increased signal for the absolute molecular intensity, M^{+}.[24]

The remaining parameters mostly affect the lateral resolution of the chemical intensity images, although alterations to the nebulizing gas flow will have effects on resolution as well as signal intensity. Increasing the flow, or pressure, will result in smaller and faster droplets, which will improve resolution and signal. This also enhances the desolvation of droplets. However, when the pressure is to high, the solvent can evaporate before impacting the sample, therefore decreasing ionization efficiency for certain molecules (ie, peptides and proteins). High gas pressure can also impair the sampling efficiency of the mass spectrometer. The high velocities of impacting solvent can also cause damage to the sample surface. At the other end, low nebulizing gas pressure can lead to improper Taylor cone formation, as well as larger droplets which can lead to extensive wetting of the sample surface and diffusion of the analyte of interest into areas it would normally not be found. This effect can also occur when the solvent flow rate is too high. When the solvent flow rate is too low, the droplets produced are too small and have the potential to evaporate prior to surface impact, similarly to when the nebulizing gas pressure is too high.

Alterations to solvent composition can produce effects relating to resolution, signal intensity, sample degradation, depth profiling, and even selective ionization by means of reactive ionization, commonly known as reactive DESI. Green et al. have explored the effects of solvent composition on signal intensity and resolution by means of assessing the spot size (or erosion diameter), solubility, and ionization efficiency.[25] A variety of organic solvents (acetonitrile [ACN], methanol [MeOH], isopropanol [IPA], ethanol [EtOH]) with varying organic:water ratios were tested. It was discovered that as

the organic content of the solvent increases, the erosion diameter decreases linearly, increasing resolution. With higher water content, the erosion diameter is much larger, as well as elongated with dendritic protrusions, thus decreasing resolution. This effect can be attributed to a few factors. Firstly, the solubility of the analyte with the chosen solvent will affect the erosion diameter. The higher the solubility of the analyte, the smaller the spot size will be. Secondly, the surface geometry will also have an affect. A uniformly flat surface will have smaller spot sizes, than a surface that is bumpy and has varying contact angles. A contact angle can be defined as the angle at which a drop of solvent comes into contact with a surface. A surface with high contact angles is characterized as being hydrophobic, such as PTFE (contact angles from 70°-110°). Erosion diameters are higher for these types of substrates due to diminishing surface wetting and a propensity for droplets to roll around. Lastly, the major contributing factor was presumed to be a correlation between higher organic solvent content and smaller droplets being produced. These smaller droplets having higher velocities are perhaps aiding the formation of a more focused electrospray jet.

The standard general solvent used in most DESI experiments is MeOH: H_2O (1:1). Using a balanced solvent is more practical when analyzing multiple classes of molecules as well as complex surfaces such as tissue samples and natural products. This solvent system is known to have a sampling depth of about 12μm, however it also causes damage to the sample. This is a huge problem in medical applications of tissue analysis where a subsequent experiment (most commonly immunohistochemical staining) is required for diagnostic validation. This lead to the discovery of "morphologically friendly" solvents described by Eberlin et al.[26] Using a solvent system composed of N,N-dimethylformamide (DMF) and other organic solvents (ACN, EtOH, tetrahydrofuran [THF], chloroform [$CHCl_3$]) (1:1) showed no signs of damage when analyzing a 15μm thick mouse brain tissue section. These solvent systems even have larger sampling depths (>50μm) than the standard MeOH:H_2O. The lack of damage seen with these solvent systems is due to the insolubility of the cellular and extracellular proteins, thereby preserving tissue morphology. When using MeOH:H_2O (1:1), the cellular proteins

become solvated, reducing the physical integrity and increasing the susceptibility to damage from impacting solvent droplets.

Difficulty can arise in cases when standard solvent systems used fail to efficiently ionize an analyte or even produce a signal. These circumstances can be overcome by adding specific reagents to the solvent, which will derivatize the analyte to increase ionization efficiency. Reactive DESI has been used to increase the detection of cholesterol, steroids, cyclic acetals, cis-diols, and esters.[9] This advantage can be exploited due to the increased rapidity of the reaction occurring in the charged secondary microdroplets. As the droplets evaporate, the pH can increase, or decrease, rapidly, which can accelerate acid/base catalyzed reactions.[27]

DESI is a remarkable innovative tool leading research in the 21st century. Capable of analyzing surfaces in open air environment without the need to house the sample in a vacuum, like its predecessors SIMS and MALDI. This allows the investigator more freedom in monitoring the sample as data is being acquired, as well as enabling the ability to perform changes to the experiment. This freedom also facilitates the possibility of performing MS/MS experiments directly from the sample, simply by moving the sample to a desired area under the spray tip. The sample also requires no pretreatments prior to analysis. Unlike with MALDI however, where an absorbing matrix must be applied to the sample for ablation and to shield the sample from the extreme energy of the laser. This is not required with DESI since there is very little energy imparted onto the analyte. Most samples are perfectly ready for analysis in their native state, however sometimes washing with solvents to remove an impenetrable layer or imprinting on porous material is necessary to improve signal intensity. These preparatory measures are quite simple and quick, and are not time consuming. All these factors attribute DESI to being a high-throughput, soft technique, with minimal fragmentation, acquiring data within seconds and completing 2D images within minutes (depending on operational parameters and sample size). DESI can be applied towards the ionization of polar and non-polar compounds ranging from small hydrocarbons and drugs, all the way up to larger molecules such as peptides and some proteins. Its versatility also allows for

production of positive and negative ions. It is also considered to be a highly sensitive technique with the ability to detect pure compounds within the sub-nanogram range.[28]

DESI is relatively easy to pick-up, yet difficult to master. Although, having numerous advantages over other mainstream techniques, it's not quite perfect. Due to the low energy of the charged solvent it is quite difficult to ionize large molecules like proteins. Analyses are typically limited to molecules of ~2000Da. Resolution is also limited by the sprayer tip size. The resolution of DESI experiments is commonly 200-250μm, but can go as low as ~40μm, depending on operational parameters.[9] It's easy to see why so many fields of research have adopted DESI to solve their problems. It is a great exploratory tool, which allows us to visualize the world in a whole new perspective.

Recently researchers have been using DESI for the detection of trace agrochemicals in foods.[29] Their repertoire included 16 different chemicals, which could be classified as being a pesticide, insecticide, herbicide, or fungicide. Analyses were performed on fruit and vegetable skin, which had been previously untreated, as well as on acetonitrile extracts of the produce. The researchers were able to detect all 16 of the agrochemicals in market purchased fruits and vegetables, in positive ion mode, using DESI-MS. The detection limit for these chemicals in the acetonitrile extracts ranged from 1-90pg, depending on the agrochemical. The composition of the matrices extracted from the produce will have varying effect on the ionization, for example a tomato will have a higher water content than an orange, which will have more organic matter. They then took it one step further and attempted to quantify one of the trace agrochemicals, imazalil, within the fresh foods extracts using an isotopically labeled standard. The results obtained using DESI-MS were in agreement with a confirmatory method using LC-MS, with a relative standard deviation of less than 15%. The results obtained from this study were also in accordance with the European Union pesticide regulation requirements; all limits of detection were below the maximum residue limits for pesticides in foods.

DESI has also been applied towards understanding and investigating the human body's interactions with biocompatible materials used in implantations, such as synthetic replacements for living tissues. The investigation focused on the processes occurring to a transplanted vascular graft that had been removed two years after implantation.[30] The imaging capabilities of DESI were employed to detect lipid species that might have made their home within the selected biocompatible material. Being conscious of the interaction occurring within implanted materials might help doctors monitor the long-term effects of the body on the physical integrity of these structures, which could lead to better materials being used as well as timely replacements if needed. The sample being analyzed was a polyethylene terephthalate (Dacron) InterGard® vascular graft. Cross-sections of the graft were collected in 35μm thickness. Analyses were performed in both negative and positive ion mode. They were able to detect cholesterol (through reactive DESI) as well as varying lipids within the inner walls of the graft. Within the inner wall was a structure similar to arterial plaque. They compared their results to those reported for the analysis of a cross-section of a human atherosclerotic artery, and were able to detect almost all the same lipids identified, not only on the graft but also embedded within. They discovered that the graft, only being in the patient for 2 years, had already succumbed to atherosclerosis throughout quite rapidly. They suppose that this process of accumulation within the graft must be a consequence of the material used, and suggest DESI-MSI as an investigative tool for the monitoring of long-term degradation and efficacy of implanted biomaterials.

The field of forensic investigation has also seen numerous applications involving the use of DESI-MS.[2] Some common applications have been in the detection and identification of explosives and residues on varying surfaces such as paper, metal, glass, and clothes. The ability of ambient ionization to analyze samples without pretreatments has facilitated the application of DESI-MS towards the detection of counterfeit drugs and pharmaceuticals in simple and complex matrices such as in pills, gels/creams, patches and solutions. DESI has also been used in ink and document identification purposes, such as ink matching in order to certify authenticity or determining whether the document had one or multiple composers. Latent fingerprint analysis has also been demonstrated to

yield complementary data for forensic investigators. By producing ion intensity images of the latent fingerprints, researchers have access to the individual's unique fingerprint pattern, as well as any chemicals with which they might have come in contact with, thus being able to possibly link a suspect to a criminal activity or crime scene, or towards the suspicion of interaction with illicit substances.

The ensuing report focuses on utilizing DESI-MS in two different fields of research. The first application applies to forensic investigations using DESI towards the analysis of sexual assault evidence such as condoms, lubricants, and their residues. Having access to this information might be able to help law enforcement in their investigation into sexual assaults. The presence of residues associated with condom or lubricant use might be able to help persecutors link a suspect to a crime or aid in providing more information in recreating a crime scene. This investigation had previously been reported for a comparable ambient ionization method, direct analysis in real-time (DART). Both techniques are ambient; therefore the sample has no pretreatments and is exposed to atmospheric conditions. This investigation was pursued in order to identify whether this type of research cold be replicated with another ambient technique. The ionization process in DESI differs from DART wherein a charged liquid solvent is sprayed onto the sample to induce ionization. DART utilizes a charged gas beam to cause ionization. DESI also differs from DART by having the ability to produce ion intensity images, which adds another dimension of information. If latent fingerprints are found at the crime scene or on items associated, it can be analyzed with DESI-MSI, and an image of the print can be created along with any chemicals or residues that the individual has come in contact with.

The second application of DESI revolves around the analysis of biological tissue, by producing two-dimensional ion intensity maps. Most applications of tissue analysis with imaging techniques, ambient or not, have been centered on small biopsies, excised areas of interest, or organs belonging to small organisms. It's rare to find a report of an analysis of a whole organism, especially a vertebrate. The larger the sample is, the more time it will take to perform the analysis. In the medical research world, the organism used

the most for experiments and pharmaceutical design is the mouse or rat. These vertebrates are relatively large, and if analyzed using an imaging technique, it could take 12-24 hours depending on the sample size. Zebra fish (*Danio rerio*) are a relatively new vertebrate organism gaining popularity within the medical, biological, and biotechnological industries. They're relatively small, between 2-4 cm in length, and whole body analysis times can be as fast as 30-40 minutes, depending on sample size and operational parameters. DESI-MSI also requires no sample pretreatments, unlike other imaging techniques such as MALDI, which require matrix application and ionization to occur within a vacuum. This application of DESI-MSI is a preliminary study demonstrating the potential for this technique in analyzing whole vertebrate organisms, specifically the zebra fish, to establish this organism as a new model vertebrate for future biological research in potential drug design and metabolite monitoring, toxicity bioaccumulation, and cancer studies.

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Chapter Two – Analysis of Sexual Assault Evidence

M. F. Mirabelli, A. Chramow, E. C. Cabral, D. R. Ifa. Analysis of sexual assault evidence by desorption electrospray ionization mass spectrometry. *Journal of Mass Spectrometry*. **2013**, 48, 774-778

Alexander Chramow

- Performed analysis and data acquisition for condoms and lubricants.
- Performed MS/MS experiments for compound identification.
- Detected condom brand identification based on relative abundance of compounds.
- Created images of chemical structures.

Mario F. Mirabelli

- Performed analysis and data acquisition for condoms and lubricants.
- Performed MS/MS experiments for compound identification.
- Detected condom brand identification based on relative abundance of compounds.
- Performed latent fingerprint experiment.

Chapter Two – Analysis of Sexual Assault Evidence

In the world of forensic investigations the suspects being investigated are small, sometimes microscopic, fragmented and invisible to the human eye. The investigators responsible for these analyses require tools and instruments to piece together the clues found at a crime scene to gain a deeper understanding of the scenario at hand. What took place here, how did it happen, were any objects involved, and most importantly who committed the crime. DESI is perfect for use in forensic research, shedding light on clues and giving investigators a glimpse into an undiscovered world. Evidence found at a crime scene is extremely important and must be handled with care to preserve its native state. Some common practices for analysis may involve the destruction of evidence. This may occur during extractions or from using separating instruments such as liquid or gas chromatography. DESI brings many advantages and advancements to forensic investigations. Sample integrity can be maintained since the ionization method is fairly soft, resulting in minimal degradation depending on the operational conditions. This extends the longevity of the same piece of evidence to be tested under successive experiments. DESI is also a very reliable, high-throughput, fast technique capable of analyzing a series of samples within seconds across a broad mass range. DESI analysis times are so quick; it could even be used as an on-site investigative tool if coupled with a portable mass spectrometer. Not every piece of evidence will be the same, or even have the same characteristics. Depending on the type of crime being investigated, law enforcement could be dealing with a ripped piece of clothing, writing on a document, traces of drugs/explosives, fingerprints, or even biological traces (blood, skin cells, semen, etc.). From molecularly small to large, solid or liquid, whatever the evidence may be, DESI is the tool for the job.

Forensic researchers acknowledge the efficiency and ease of compound identification capable by DESI. Cotte-Rodríguez et al. have studied the abilities of DESI to detect trace amounts of various commonly used explosives such as TNT, RDX, HMX, PETN and their plastic compositions C-4, Semtex-H, and Detasheet on a range of different surfaces (glass, paper, metal, polymers).[1] Using DESI they were able to detect

TNT and RDX at 10fg and 1pg, respectively, deposited onto paper. They were even capable of identifying trace explosives when masked with an interfering matrix such as gasoline, glass cleaner, bleach, and vinegar with the aid of MS/MS identification.

Other than identifying trace compounds used in chemical warfare, explosives manufacturing, drug trafficking, hazardous materials, and any crime that fits a yes or no type of conviction, ambient ionization techniques can also aid forensic investigators in reconstructing a crime scene or understanding a criminal's *modus operandi*. This application can be experienced when dealing with sexual assault evidence. Its been reported that in 2006 over 300,000 women experienced forced sexual assault in the United States.[2] A large majority of sexual assault offenses are underreported.[3] An analysis of five different countries (Australia, Canada, England, Scotland, and the United States) identified that only 14% of sexual assault victims actually reported the offence.[4] Those that do get the attention of law enforcement lack sufficient evidence to create a case, and only 30% make it to court. From those cases that do go to court most of them will lack probative evidence to link a suspect to the crime unless they have substantive evidence such as DNA, and even then its not enough to firmly determine that the offence actually occurred. Once in court, 20% of the cases will end in adjudication, and 12.5% of the perpetrators are convicted of some sort of sexual assault crime. In the end only 6.5% of perpetrators were actually convicted of the originally charged offence.[4] In the criminal world, sexual assaulters are becoming more aware of the implications of leaving behind a biological trace (saliva, blood, semen),[5] and condom use amongst them is on the rise. A study was conducted which identified a link between alcohol consumption of perpetrators and condom use. Almost half of the subjects admitted to alcohol consumption prior to committing a sexual assault. And from those individuals, only 41.2% admitted to never using a condom during aggressive sexual assaults.[2] If at a crime scene a condom is found, part of the wrapper, or even a fingerprint containing lubricant residues, these pieces of evidence could be used to support a victim's claim that sexual assault has been committed, the extent of the crime and even link a possible suspect. It has previously been reported that condoms have successfully been used as evidence to link perpetrators to sexual assault crimes.[5]

Investigative research of this quality has previously been conducted by Shepard et al. In their paper they discuss the use of another ambient ionization technique, direct analysis in real time (DART), to analyze sexual assault evidence such as condoms and latent fingerprints. They were looking to identify compounds related to lubricants, spermicide, manufacturing and other trace compounds for the potential use as probative evidence in reaching convictions for sexual assault perpetrators.[3] DART is an ambient ionization source, similar to DESI, which uses a charged flow of gas (helium), in replace of solvent, to desorb and ionize sample analytes for detection in a mass spectrometer. Samples can be in the form of liquid, solid, or gas.[6]

DART is similar to DESI in respect that they both operate in ambient conditions, however they differ in their ionization mechanisms. DESI relies on liquid-phase processes and wetting of the sample surface. On the other hand, DART depends on gas-phase ionization processes. The ion source for DART consists of a tube structure (figure 2.1) in which the reagent gas (usually helium, but sometimes nitrogen or argon) is fed into. In this first chamber, a corona discharge is produced from a needle electrode and a perforated disk electrode. This discharge produces ions, electrons, and excited species. This plasma then passes through a second perforated disk in order to remove any cations. The passing gas beam is then heated (50-550°C) in the next chamber, and then passes through a final grid electrode, which removes any anions and electrons. Thus the emanating gas will only contain electronically excited neutral species, which then flow into the reaction zone. These excited species then undergo a cascade of gas-phased reactions, such as Penning ionization, with molecules present in the atmosphere, which can include nitrogen, oxygen and water vapor. These reagent ions then interact with the sample analytes to cause chemical ionization. The sample is introduced to the ionizing gas beam trough a gap that exists between the source and spectrometer inlet. For the production of positive ions, the second and final disk electrodes are set to a positive potential, and for the production of negative ions the potential for the second and final electrodes are set to negative. DART-MS can be limited in the types of analytes (*m/z* 50-1500) which can be ionized since the process depends on analyte volatility and thermal

stability. The gas beam can be heated to enhance the ionization of less volatile molecules.[7]

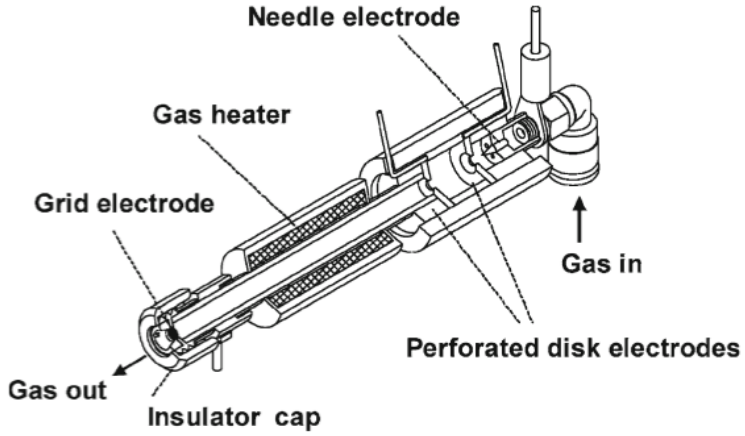

Figure 2.1 – Schematic of DART ion source.[7]

In their study they analyzed three condoms, Lifestyles®, Trojan®, Lifestyles SKYN®, and a fingerprint saturated with lubricant impressed onto a condom wrapper and a glass slide. The mass spectra produced by these analyses detected various compounds associated with manufacturing processes such as, a germicide/ emulsifier/ dispersant compound, rubber anti-oxidant, UV stabilizer, cosurfactant, and catalysts. They also detected the spermicide nonoxynol-9 on Trojan® brand condoms, which was the only one to contain the spermicide. What they discovered was that each condom brand had its own unique relative abundance of these common condom-manufacturing compounds. What they propose from these findings is that these unique formulations for each condom brand could be used to identify an unknown condom, or condom residue for use as evidence in aiding law enforcement. In previous sexual assault cases, the presence of nonoxynol-9 has been used as probative evidence. What the researchers postulate from their results is that any trace compound related to the feasibility of sexual assault be it condoms, lubricants, additives, plasticizers, anti-oxidants or other compounds related to the manufacturing process should be considered probative evidence for use in convictions of sexual assault crimes.

Since DESI is fairly similar in its *modus operandi* compared to DART, it seemed like an interesting endeavor to see if the results could be replicated using another ambient

technique. DART-MS and DESI-MS have both gained notoriety through investigating and analyzing similar samples, ranging from natural product analysis and identification of pesticides to forensic and public safety concerns in detecting chemical warfare agents, explosives and illicit substances on various surfaces such as clothes, luggage, and bank notes.[7] DESI is similar to DART in that analysis times are fast, occur in ambient conditions, and require no sample pretreatments prior to analysis. DESI also has an advantage over DART since it has the capability to produce two-dimension ion intensity maps. This proves to be very useful when encountering latent fingerprints at crime scenes.

Table 2.1 – Characteristics of DESI-MS and DART-MS.

Parameter	DESI-MS [8]	DART-MS [7]
Mass range	Limited by mass spectrometer	*m/z* 50-1500 (dependant on analyte volatility)
Ions produced	Positive & negative	Positive & negative
Types of analytes	Polar, non-polar, small to large	Polar, non-polar, small to medium

The intent of this study was to identify if DESI, another ambient ionization technique, could be used for the analysis of sexual assault evidence, such as condoms and lubricants, for the detection of trace compounds. The total amount of condoms analyzed was expanded slightly for this study (Trojan Enz®, Durex Love®, Lifestyles®, Lifestyles SKYN®), as well as the inclusion of a few personal lubricants. During the analysis several compounds were detected which were indicative of the presence of a condom and could aid in condom brand identification. The analysis of a latent fingerprint was also emulated, in this case a two-dimensional ion intensity image was produced to demonstrate the ability of DESI-MSI to acquire the data required for the identification of an individual as well as any chemical information regarding substances which the individual has come into contact with.

Experimental

Materials

The solvents used during experiments were methanol, ammonium acetate, and formic acid. All solvents were purchased from Fisher Scientific Canada (Whitby, ON, Canada). The condoms and lubricants analyzed were Trojan Enz®, Durex Love®, Lifestyles®, Lifestyles SKYN® and Wet Platinum® Premium Silicone lubricant™, Liquid Silk® Personal lubricant, K-Y® Personal lubricant, respectively. Condoms and lubricants were purchased from the campus pharmacy and a local grocer. Microscope glass slides used for lubricant analysis were purchased from Bionuclear Diagnostics Inc. (Toronto, ON, Canada).

Sample Preparation

For each analysis a new condom was removed from a fresh package using nitrile gloves to avoid contamination. It was then unraveled and a ~3cm section was excised from the centre. The condom section was then immediately placed on the DESI mounting plate and adhered with tape. Both the inside and outside of the condoms were analyzed. For lubricant analysis, a drop of unknown volume of lubricant was placed onto a glass slide, and subsequently analyzed by the mass spectrometer.

Mass Spectrometry

All data was collected using a Thermo Scientific LTQ linear ion trap mass spectrometer (San Jose, CA, USA) equipped with a lab-build automated DESI ion source. Data was acquired in the positive ion mode ranging from *m/z* 50-1500. Operational parameters were as follows: spray voltage, 5kV; MS injection time, 250ms; 2 microscans averaged; nitrogen sheath gas, 100psi; incident angle, 52°; tip-to-surface, 2mm; tip-to-inlet, 5mm; collection angle, 10°; solvent flow rate, 2.5µl/min.

Results and Discussion

Preliminary experiments revolved around determining an optimal solvent for the analysis of condoms and lubricants. Various solvent combinations were tested such as 9:1 methanol:water, 9:1:0.05 methanol:water:formic acid and 9:1:0.05 methanol:water:ammonium acetate. Each of these solvents produced completely different spectra for the same condom sample. Using pure methanol as the solvent yielded the best most stable results. Using DESI it was possible to consistently detect three of the manufacturing/trace elements associated with condoms that were also detected using DART. These compounds are listed in table 2.2.

Table 2.2 - List of condom manufacturing compounds detected in positive ion mode using DESI.

Compound Name	*m/z*	Properties	Compound I.D
N-methylmorpholine	102	Accelerator in rubber manufacturing	A
N-octylamine	130	Germicide/emulsifier/ Dispersant/ or lubricant	B
N,N-dibutyl formamide	158	Cosurfactant / catalyst	C

Each condom analyzed produced a unique mass spectrum associated with it. This should be expected since every condom brand has its own unique formulations for manufacturing, applying various ratios of common manufacturing compounds during the production process. However, the unifying factor within all the condoms was that they each contained the three compounds listed in table 2.2 (except for Durex Love® which only contained the first two). The identities of these three compounds were confirmed with MS/MS identification and compared with those derived from the DART study conducted by Shepard et al. The MS/MS spectrum for N-methylmorpholine (Figure A2) (MS/MS: *m/z* 102, 74, 60, 56) shows a loss of CO, resulting in fragment ion *m/z* 74. The identification of N-octylamine (Figure A3) (MS/MS: 130, 74, 57) was confirmed by the fragment ion *m/z* 74, resulting from a loss of C_4H_8 (56 Da). And finally, the structure of N,N-dibutyl formamide (Figure A4) (MS/MS: *m/z* 158, 116, 102, 57) is confirmed by the loss of C_4H_8 (56 Da), which can break off of the N-C bond. The MS/MS characterization

of these compounds was performed by increasing the CID energy until a stable signal was attained. The exact value and units of the energy is not known since it was reported in an arbitrary value used by the instrument.

There was very little difference when analyzing the inside and outside of the condoms. It was discovered that the inside contained a slightly higher abundance of N-octylamine and N,N-dibutyl formamide. The relative abundance of N-methylmorpholine was relatively constant from the inside and outside.

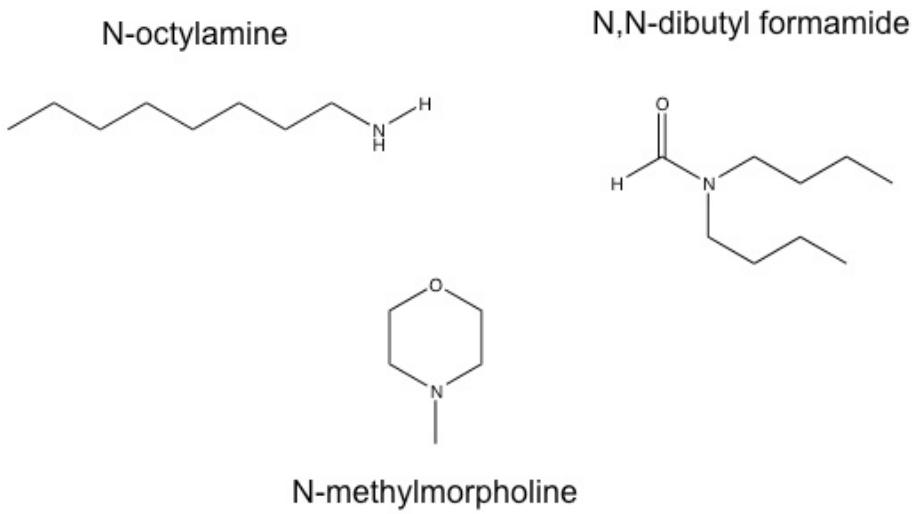

Figure 2.2 – Chemical structure of manufacturing compounds detected on the condoms by DESI-MS.

It is these three compounds (figure 2.2) that represent the basis of differentiation between condom brands. In each spectrum it is possible to determine the ratios of the relative abundance of these three molecules in order to differentiate one condom brand from another, or to identify an unknown condom sample. For example the four condom brands used in this experiment: Trojan Enz®, Durex Love®, Lifestyles®, and Lifestyles SKYN®, could all be differentiated from each other based on their unique relative abundance of the three manufacturing compounds identified as well as the unique formulation of polymeric substances which make up the condom. By looking at the value of the relative abundance collected from the mass spectrum for these three compounds, it is possible to determine an identifying ratio for each condom brand. Each of the relative abundances for the three compounds yields the following ratios for the condoms Trojan

Enz®, Durex Love®, Lifestyles®, and Lifestyles SKYN® (Table 2.2, A:B:C): 1:7:3, 1:25:0, 2:5:1, and 1:1:3, respectively. The mass spectra recorded for each condom brand remained consistent. In the end, each brand, and sub-brand of condom produced unique mass spectra based on the manufacturing formulations, polymeric substances, and inclusion of spermicide, or lack there of.

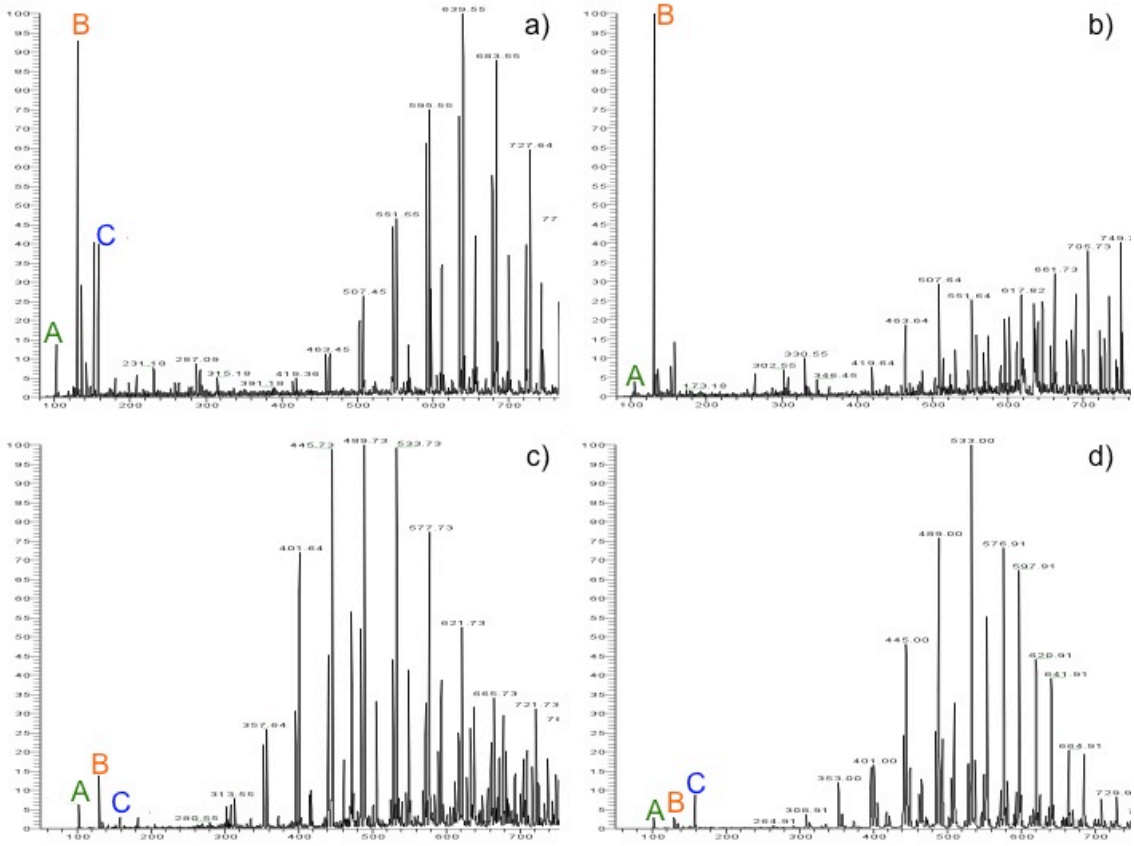

Figure 2.3 – Positive ion MS Spectrum of each condom brand, identifying common manufacturing compounds from Table 2.1. (a) Trojan Enz®, (b) Durex Love®, (c) Lifestyles®, (d) Lifestyles SKYN®

The analysis of lubricants demonstrated similar results. Each brand of lubricant contained a unique formulation, which in turn produced a unique mass spectrum. Each of the lubricants analyzed were composed of different bases. The Wet Platinum® lubricant is based on polydimethylsiloxane, Liquid Silk® Personal lubricant is based mostly on propylene glycol and some polydimethylsiloxane, and K-Y® Personal lubricant is based on hydroxyelthyl cellulose. Further work needs to be done in order to identify the compounds detected in each lubricant's mass spectrum. However, the same

"fingerprinting" process can be applied towards lubricants in identifying one from another. Depending on what their base is composed of and which manufacturing compounds are used during processing, unknown lubricant residues could also have the ability to be identified based on their unique mass spectra.

Latent fingerprints were also analyzed after handling a condom sample. Fingerprints were made on various surfaces such as glass, paper, and metal, and then analyzed with DESI to create an ion intensity map (figure 2.4). It was detected that images created from fingerprints blotted on glass yielded the best results in terms of signal intensity for low and high mass molecules. Those on paper had better signal for lower mass molecules, while metal had the preference for high-mass molecules.[9]

Figure 2.4 – DESI-MS of latent fingerprints blotted on different materials after touching a Trojan Enz® condom. Surfaces: (A, D, E, and H) glass, (B and F) metal, and (C and G) paper. Fingerprints (D and H) were analyzed after 1 month aging. Panels A–D show the distribution of the ion m/z 463 (sodiated nonoxynol-9 with n = 5), and panels E–H show the ion m/z 727 (sodiated nonoxynol-9, n = 11) on the same surfaces, respectively.[9]

Conclusion

It has been demonstrated that DESI has the necessary capabilities for the analysis of sexual assault evidence, such as condoms, lubricants, and their residues, for use as probative evidence in sexual assault crimes. The trace manufacturing compounds associated with these products could be used to identify the condom or lubricant at the crime scene and potentially link an action to a crime. Samples can be analyzed directly without the need for any pretreatments or destruction like other conventional and surface analysis techniques, leaving the potential "evidence" in its native state. This can allow for further testing such as DNA profiling. DESI analysis times are very quick, yielding real-time data, and could even exert the possibility of being applied in the field with mini portable mass spectrometers. This study has only explored the identity of a few condom brands, however for this process to become a valid method of unknown identification there needs to be an extensive library created, drawing from multiple brands and sub-brands. Since DESI is also an imaging technique, it has the ability to create fingerprint images found at crime scenes. This information becomes extremely important when it is found combined with the presence of condom or lubricant residues. The potential for convicting individuals suspected of committing sexual assault increases when there is a fingerprint linking the perpetrator with contact to the evidence. This study represents preliminary results associated with identifying unknown condoms and lubricants in a controlled lab setting. Further work needs to be done assessing the viability of the evidence as it is in the real world, enduring the elements as well as degradation over time.

Chapter Two References

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Chapter Three – Whole-Body Imaging of Zebra fish (*Danio rerio*)

A. Chramow, T. S. Hamid, L. S. Eberlin, M. Girod, D. R. Ifa. Imaging of whole zebra fish (Danio rerio) by desorption electrospray ionization mass spectrometry. *Rapid Communications in Mass Spectrometry*. **2014**, 28, 2084-2088

Alexander Chramow

- Sample handling and preparation:
    - Creation of embedding medium
    - Slicing of Zebra fish
- Set up experiments for two-dimensional ion image creation
- Performed *in situ* MS/MS experiments
- Determined identity of lipid species analyzed
- Created ion intensity images (figure 3.2) of zebra fish for various lipids and bile salt

Tanam S. Hamid

- Sample handling and preparation:
    - Creation of embedding medium
    - Slicing of Zebra fish
- Set up experiments for two-dimensional ion image creation
- Performed *in situ* MS/MS experiments
- Created figure 3.1, overview of zebra fish analysis process

Chapter Three – Whole-Body Imaging of Zebra fish (*Danio rerio*)

Modern western medicine has always focused on the longevity and preservation of the human species. However, most discoveries that have enriched our lives have been made possible through the sacrifice of smaller organisms, possessing similar characteristics to humans. The standard model has been the use of genetically modified mice, which are engineered to mimic symptoms of diseases and disorders generally afflicting humans. These mice then get put through treatments and medications and are monitored for the ensuing effects. It is of utmost importance to the researcher to understand what is occurring at the molecular level during experimental treatments. If a researcher is working with a tumorous mouse and it is receiving an experimental drug treatment, how is the researcher to know if the drug is affecting the desired area, what is it metabolizing into, is the drug working, has the tumor reduced, etc. Ambient imaging mass spectrometry has the capabilities of providing answers to all these questions and more.

Most of the conventional established tissue analysis techniques don't provide enough information. Some techniques provide the location of drugs without molecular structure, such as immunohistochemical staining. Other techniques, such as liquid chromatography, require laborious separation processes that trade off localization for molecular information. However, this requires a sample to be homogenized and then analytes extracted for analysis. The industry standard for analyzing drugs in tissues is whole-body autoradiography (WBA). This technique provides quantitative and spatial information, however, the analyte in question must be radioactively labeled, which is time consuming and costly. Most importantly it is the radioactive label that is detected not the analyte, which makes it difficult to distinguish between a drug and its metabolites.[1] Researchers have demonstrated that DESI has the ability to analyze tissues to solve a variety of issues such as, cancer identification,[2] localization and identification of lipids,[3] and monitoring exogenous compounds, such as drugs, and their metabolites.[1] Other comparable methods simply require too much time invested in sample pretreatments such as matrix application with MALDI, and the use of vacuums. DESI is

much more effective than most conventional techniques since it can provide both spatial and molecular information regarding analytes. Analysis times are quick, there is little to no sample pretreatment, and there is minimal damage rendered to the sample leaving the possibility open for additional experimentation.

Most of the MSI research implemented on biological tissues have either been excised from rodents (rats or mice) or generously donated by humans. However, there is a new organism that is emerging as a vertebrate model for understanding and combating human diseases as well as drug discovery.[4] Zebra fish (*Danio rerio*) have been receiving increased attention as an alternative to using mice for medical inquiries and drug development. These fish are small, have rapid generation cycles, and can be maintained at a lower cost than mice.[5] Zebra fish have been gaining popularity since the 1990's due to the optical clarity of their embryos and its suitability towards chemical genomics. Pioneering research involving genes and mutations of the zebra fish have lead to discoveries about physiology, behavior, and human dysmorphologies.[6] This caliber of work and excitement helped progress the complete sequencing of the zebra fish genome, as well as, acceptance into the biotechnology industry. Since then, zebra fish have been used in studies for embryological and human development and disease and for toxicological purposes.

In most MSI experiments it is common for the researcher to study a specific area, or organ one at a time. This is possibly due to signal suppressing effects caused by adjacent lipid groups, or perhaps due to the long analysis times when acquiring data for a two-dimensional ion intensity image. To illustrate analysis times for varying sample sizes, take for example, the average length of a mouse, which can be up to 7 cm or longer depending on age, and rats, which can be up to 2 to 3 times longer. By contrast, the average size of a zebra fish in captivity is less than 4 cm in length. Depending on your experimental conditions, it could take up to 3 minutes (or more) to complete one scan across a 3.5 cm long zebra fish. It could take up to 5.6 minutes for an average mouse (7 cm), and >13 minutes for the average rat (17.5 cm). And once you begin to factor in the second dimension for the whole body analysis, data acquisition times can run into a

couple of hours for a small fish, and up to an entire day for large rats. When considering performing an experiment on this scale, size is an important variable, which will effect a researchers time and cost management.

The following study explores the capabilities of ambient ionization for the whole body analysis of zebra fish using desorption electrospray ionization mass spectrometry. Ambient techniques facilitate the analysis of biological tissues by eliminating the need for sample pretreatments or the use of vacuums, leaving the sample relatively unchanged from its native state. It also allows the performance of *in situ* MS/MS ion characterization directly from a tissue sample. The potential for using the zebra fish as a model vertebrate organism will be investigated by creating two-dimensional ion intensity images of lipids, fatty acids, and bile salts. These ions represent areas of interest such as the brain, body fat, and gall bladder/liver. Having the capability to analyze an entire organism will allow researchers to monitor how external or internal modifications affect the organism as a whole, rather than being constrained to analyzing one area, organ or system at a time. This type of analysis could have future applications involving the zebra fish studying drug design or toxicology, by creating 2D ion images to monitor the distribution of endogenous compounds and their metabolites. The analysis of lipids can also prove to be very insightful. The field of lipidomics is growing thanks to recent advances in mass spectrometry. These investigators are concerned with the structure, function, and interactions of cellular lipids and any differences that might occur when drifting from normal biological function. By monitoring any change in lipid profile expression across a whole organism, researchers might be able to learn more about cellular interactions across multiple systems.

Experimental

Sample and Chemicals

Euthanized male adult zebra fish were kindly donated by Dr. Chun Peng's research group (Biology dept., York University). Carboxymethyl cellulose used to create moulds was purchased from Sigma-Aldrich Canada Co. (Oakville, Ontario). Solvents used were acetonitrile (ACN) (Optima LC/MS) from Fisher Scientific Company (Ottawa, Ontario), N,N-dimethylformamide (DMF) (HPLC grade) from Sigma-Aldrich Canada Co. (Oakville, Ontario), and Methanol from Fisher Scientific Canada (Whitby, ON, Canada).

Zebra Fish Tissue Preparation & Sectioning

Prior to cryosectioning, the euthanized zebra fish were placed in disposable plastic rectangular frame/ moulds in order to protect them from deformation and damage during sectioning. These moulds were first filled halfway with a carboxymethyl cellulose (CMC) solution; the fish were then placed on top and the moulds filled in with more CMC. The filled moulds were placed immediately in the refrigerator at -8 °C for chilling overnight. The solution of carboxymethyl cellulose was made by mixing CMC with heated distilled water until it formed a saturated paste. The frozen CMC blocks were removed from the plastic frame and continuously sectioned into 20 - 60 μm thick sagittal sections at -17 °C using a Shandon Cryotome FE and FSE (Thermo Fischer Scientific, Nepean, ON, Canada). The frozen fish slices were thaw mounted on microscope glass slides and kept in the freezer until use. The fish slides were then dried in the lab in the open air for 30 minutes prior to analysis by DESI.

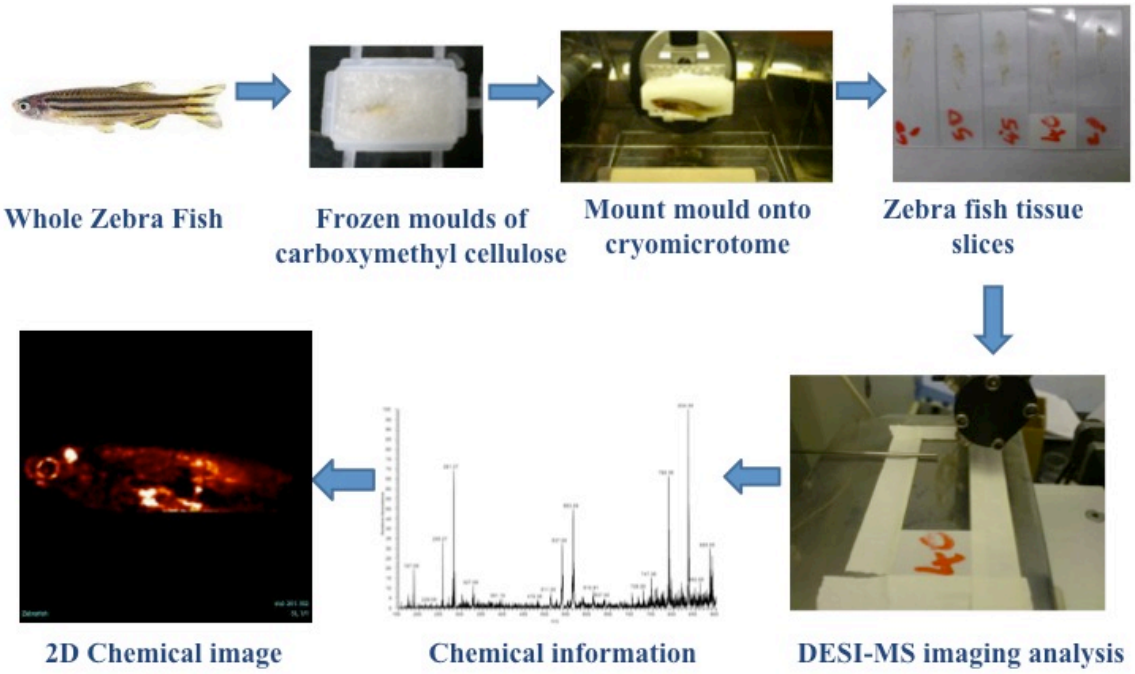

Figure 3.1 – Overview of zebra fish tissue processing and analysis.

DESI Imaging Experiments

Imaging was carried out using a lab-built prototype DESI ion source. The DESI spray tip was positioned approximately 2.6 mm from the tissue sample at an incident angle of 52°. The MS inlet was approximately 2 mm from the tissue sample surface with a collection angle of approximately 10°. The spray tip-to-inlet distance was 5 mm. Experiments were conducted in negative ion mode with spray voltage of 5 kV, injection time 250ms, 3 microscans averaged, solvent flow rate of 1.5 μL/min, and nebulizing gas (N_2) pressure of 100 psi. The mass spectrometer used was a LTQ linear ion trap mass spectrometer controlled by Xcalibur 2.0 software (Thermo Fisher Scientific, San Jose, CA, USA). The two-dimensional imaging experiment which produced the image in figure 3.2 was performed by programming a moving stage to scan an area of 2.5 cm in the x-direction and 0.6 cm in the y-direction. The velocity for the moving stage was 208.3 μm/s, acquiring mass spectra ranging from *m/z* 150 to 900. The 2D image acquired consisted of an array of 125 × 30 pixels, each pixel covering an area of 200 × 200 μm^2. Each pixel required 0.96 seconds to be scanned making the total scan time for the 2D image to be approximately 60 minutes (0.96 s /scan × 125 scans × 30 lines). An in-house

program allowed the conversion of the Xcalibur 2.0 mass spectra files (.raw) into a format compatible with Biomap (freeware, http://www.maldi-msi.org) creating single ion images as well as overlays of two different ion images. In order to adjust the colour contrast of the images, the rainbow colour gradient was selected and the intensity gain was normalized for all images used. The color intensities for the ions were all normalized to the same level so that one ion might not appear deceivingly more abundant than another. The fatty acid, bile salt and lipid species were identified by comparing the MS/MS data generated through collision-induced dissociation (CID) experiments with spectra acquired from literature. CID experiments were performed directly on a tissue slice by moving the sample stage in order to position an area of interest under the spray tip. The CID energy was optimized by manually ramping up the energy from 10-30 units and monitoring the ion fragments. Optimization was attained when a stable signal with multiple ion fragments were produced. The *m/z* range for the ion selection window was 1.

Results and Discussion

Sample Handling and Sectioning

Although it has been stated that when working with DESI there is no sample pretreatment involved, there is some preparative work required to be done on the zebra fish prior to sectioning and analysis. The fish must first be frozen in an embedding medium to maintain the structural integrity during cryosectioning. Most fish contain an air-filled swim bladder, that once frozen can cause deformations during cryosectioning. There are many different choices for embedding mediums such as, OCT compound (optimal cutting temperature, polymer based), TBS medium (Triangle Biomedical Sciences), carboxymethyl cellulose (CMC), gelatine, agarose, ice, and sucrose. Little work has been done on which medium endows the optimal cutting conditions. However, recent work done by Nelson et al. explores the efficiency of these embedding mediums specifically for the cryosectioning of zebra fish.[7] They discovered that the optimal embedding medium was a mixture of 5% CMC with 10% gelatine. Using this medium, they were able to reproducibly acquire tissue slices with a 16 μm thickness.

For this study, the available medium for embedding the zebra fish was carboxymethyl cellulose. When preparing the embedding medium, the amounts of water and CMC used were not measured. Instead, the viscosity of the solution was monitored until a desired paste consistency was attained. During the first attempt at preparing the medium the water was not heated. This limited the amount of CMC which could be dissolved into the water, and resulted in a harder medium resembling ice. Subsequent attempts used heated water which produced a medium that was more gelatinous.

Once the fish are frozen in the embedding medium, the next step is to slice them. Operating a cryotome is a learned skill that only comes with practice. Before slicing, it is important to determine the optimal temperature for sectioning a sample. Establishing an optimal temperature will allow for uniform optimized slices to be acquired, while minimizing slice deformation. For zebra fish, it was determined that the optimal cutting temperature was somewhere in-between -16 to -18°C. The value is reported as a range

since there were issues around controlling or maintaining a constant optimal temperature. At times the sample would become too cold or frozen, therefore it had to be removed for a short time from the cryotome in order to reduce its temperature. The frozen zebra fish blocks were trimmed down to avoid useless slicing, and then adhered to a mounting plate using a strong glue. All sections were obtained in the sagittal plane. Due to the set-up of the cryotome, it was difficult to acquire tissue slides of <20 μm without having the sample curl in on itself. Slices were collected in thicknesses ranging from 20-60 μm, which were thaw mounted onto microscope glass slides. Little to no difference was seen in analyzing slides up to ~45 μm thickness. When slices are thicker they appear "fleshy" and retain to much moisture to dry properly. A non-dehydrated sample could be blown away by the high pressure nebulizing gas or even enter the mass spectrometer. Newly collected tissue slides were kept in an insulating container with dry ice until they could be transferred to the lab freezer where they were permanently housed at -8°C. Prior to analysis, the zebra fish slides were allowed to dry for ~30 minutes, exposed to the atmosphere, at room temperature in the lab.

Mass Spectrometry and Imaging

The zebra fish slices were analyzed in both positive and negative mode. In positive mode, the whole body analysis did not yield any information. The images produced showed nothing more than background noise. In contrast, analysis in negative mode yielded various ions, such as fatty acids and lipids, and produced great quality images. It has been reported previously in the literature that phospholipids and sphingolipids ionize more easily in negative mode.[8]

Initial experiments used a standard solvent composed of methanol and distilled water (MeOH:H_2O; 1:1). Later experiments adopted a different solvent mixture of, acetonitrile and dimethylformamide (ACN:DMF; 1:1), which has shown to be a morphologically friendly solvent and allows for the potential of subsequent testing with MALDI or chemical staining. Previous experimentation with MeOH:H_2O (1:1) as a solvent have demonstrated that it is capable of sampling depths ~12μm, however it also

causes macroscopic surface damage. By comparison, ACN:DMF (1:1) is able to reach sampling depths of >50μm, and can extract lipids with high efficiency without causing morphological damage or disrupting protein content.[9]

The experiments performed in negative ion mode with MeOH:H_2O yielded spectra with lower signal than ACN:DMF. They also provided lower quality images. This could be attributed to several factors, one being that as you increase the water content of a solvent, the spray spot size will increase and it will also cause "splashing" leading to a diffusion of analytes. This diffusion will yield smudgy, pour quality images. As organic content is increased in the solvent, the spray spot size is consolidated, a splashing effect is no longer noticed, and the resolution and quality of the images is enhanced. Another factor is the solubility of the analyte in the chosen solvent. If a solvent is chosen which easily solvates the analyte, it will yield increased signal intensity, and greater quality images.[10] Perhaps ACN:DMF was more successful in solvating the lipids and fatty acids than MeOH:H_2O.

Table 3.1 – Proton affinities for solvents used in DESI analysis of zebra fish.[11]

Solvent	**Proton Affinity (KJ/mol)**
H_2O (water)	697
Methanol (MeOH)	761
Acetonitrile (ACN)	788
Dimethyl formamide (DMF)	884

The ions being generated during these experiments are mostly deprotonated species $[M-H]^-$, therefore ionization would be enhanced by a solvent that has a greater proton affinity than the analyte. When comparing the proton affinities for the solvents used, found in table 3.1, it becomes clear why one mixture produces greater results than the other. The proton affinities for the standard DESI solvent system, and the first one used, MeOH:H_2O is 761:697 KJ/mol, respectively. Compared to the morphologically friendly solvent mixture adopted afterwards, ACN:DMF which has a proton affinity of

788:884 KJ/mol. The higher proton affinities of ACN:DMF allow for enhanced ability to abstract a proton from the analytes, resulting in better signal intensity which will produce greater ion intensity images.

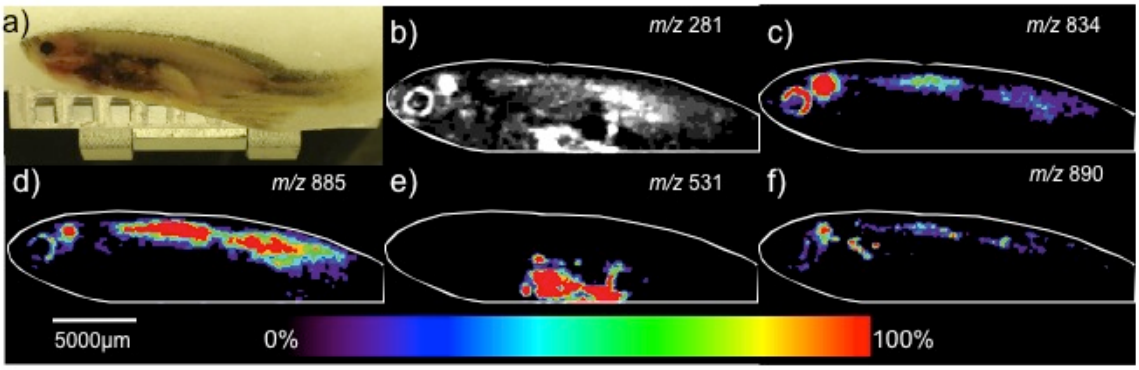

Figure 3.2 – (a) Optical image of zebra fish in CMC mould. (b-f) Images of ion distribution collected in negative ion mode from DESI-MS analysis. (b) Deprotonated oleic acid *m/z* 281, (c) phosphatidylserine (PS 40:6) *m/z* 834, (d) phosphatidylinositol (PS 38:5) *m/z* 885, (e) bile salt 5α-cyprinol 27-sulfate *m/z* 531, and (f) sulfatide (ST 24:0) *m/z* 890.

The analysis of the whole body zebra fish using ACN:DMF (1:1) as solvent yielded mass spectra providing a diverse collection of ions with fatty acids dominating the lower *m/z* range (*m/z* < 400), dimers of fatty acids and bile salt in the mid-range (*m/z* 500-600), and phospholipids and sphingolipids in the high range (*m/z* > 600). Figure 3.2 (b-f) shows the ion images created from one 45µm thick sagittal slice of a zebra fish collected in negative ion mode. Slices were taken at this thickness due to the ease of reproducibility with the chosen embedding medium. The chemical images are set next to an optical image (Figure 3.2a) of another sliced zebra fish frozen in a CMC mold, adhered to the mounting plate of a cryotome. In the optical image there is distinction between different tissue types: digestive, neuronal, muscular. Figure 3.2b represents the chemical intensity map of ion *m/z* 281, deprotonated oleic acid. Since it is found in the majority of the tissue it serves as a good background for making comparisons with other ions of interest (*m/z* 531, 834, 885, 890). Figures 3.2c-f are images of the chemical distribution of ions of interests shown in a colour gradient.

The ion of *m/z* 834 (figure 3.2c) appears localized in the area representing the brain of the zebra fish. This ion is phosphatidylserine (PS 40:6), its structure is confirmed by comparing MS/MS spectra (MS/MS: *m/z* 834, 747, 437, 419, 327, 283)

with the literature.[12] The most intense peak at *m/z* 747 [M – H – 87]$^-$ corresponds to the loss of the serine head group (87 Da), which confirms the identification as a phosphatidylserine (PS). It is reasonable to find this ion localized within the brain since the main species of lipids detected in the brain are phosphatidylserines (PS), phosphatidylinositols (PI), and sulfatides (ST). In a previous study analyzing rat brains using DESI, the ion *m/z* 834 (PS 40:6) was found with high intensity in areas correlating to grey matter, rather than white matter.[13] This indicates that this phospholipid resides mostly within the cell body, or soma, of neurons.

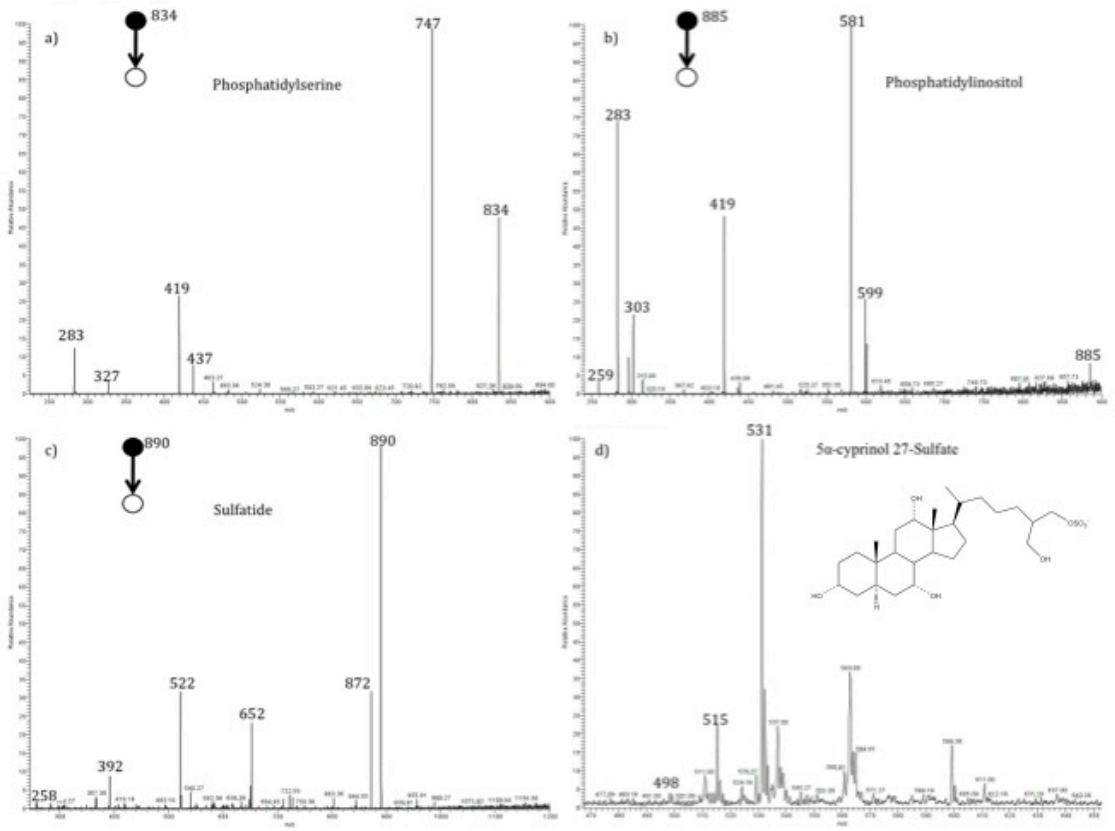

Figure 3.3 – DESI negative ion mode MS/MS spectra for ions (a) PS 40:6 *m/z* 834, (b) PI 38:4 *m/z* 885, (c) ST 24:0 *m/z* 890, and (d) negative MS spectra from *m/z* 460-660 showing bile acids and alcohols present in zebra fish bile. *m/z* 498 is C_{24} bile acid-$(OH)_2$-taurine, *m/z* 515 is C_{27} bile alcohol-$(OH)_4$-SO_4, *m/z* 531 is C_{27} bile alcohol-$(OH)_5$-SO_4.

Phosphatidylinositol (PI 38:4) is represented as ion *m/z* 885 (figure 3.2d). This assignment is confirmed by the presence of inositolphosphate (*m/z* 259) in the MS/MS spectra (MS/MS: *m/z* 885, 599, 581, 419, 303, 283, 259). Phosphatidylinositol (PI) plays a crucial role in intra-cellular communication,[12] therefore it would be logical to see this

ion in neuronal tissue, with a much greater accumulation and intensity in the area representing the spinal cord, where a lot of information is cross-linked and carried through.

Another lipid appears as ion of *m/z* 890 (figure 3.2f), and is identified as sulfatide (ST 24:0). This is confirmed by the presence of the fatty acid amide-containing ion *m/z* 392 in the MS/MS spectra[14] (MS/MS: *m/z* 890, 872, 652, 522, 392, 258). Sulfatides (ST) are a very important sphingolipid since it is present in myelin sheath and has a role in cell differentiation and adhesion.[14] The areas of the zebra fish highlighted by the distribution and intensity of ion *m/z* 890 could possibly represent the white matter of the nervous system.

The bile salt of the zebra fish was identified as ion *m/z* 531 (figure 3.2e), 5α-cyprinol 27-sulfate (MS/MS: *m/z* 531, 513, 471). This ion is specifically localized within the area corresponding to the gall bladder and possibly the liver of the zebra fish. A previous study on the evolution of bile salts has identified that at least 98% of the bile salts in zebra fish are from 5α-cyprinol 27-sulfate.[15] The mass spectra recorded for the pixels containing bile salt are comparable to those reported for purified extracts of zebra fish bile. A Stable signal for the MS/MS characterization could not be obtained for further identification.

Conclusion

DESI-MSI has proven itself to be a powerful tool in analyzing biological tissues. It has also demonstrated great capabilities for whole body analysis of zebra fish. This technique has made it possible for researchers to visualize areas of interest in 2D, such as the brain, spinal cord, and gall bladder/liver, which can be further classified by their lipid species or bile salt make-up. These markers can also be characterized and their structure elucidated through MS/MS experiments, which can be acquired directly from a tissue sample. Unlike most conventional techniques in practice, which use powerful ionization methods (MALDI) or destructive extractions (LC-MS), DESI provides a softer, less time consuming alternative with little to no sample pretreatments, leaving open the possibility for additional analysis with complementary techniques. The ability to analyze whole organisms could allow for future research on bioaccumulation and toxicological studies by exposing the fish to toxins, ionic liquids, detergents, etc. Previous studies on the zebra fish have included determining the LD_{50} (lethal dose causing 50 percent death in a population) for various toxins such as metribuzin,[16] xinjunan,[17] and endosulfan.[18] By applying the information discovered by these studies, the zebra fish could potentially be monitored for the distribution of drugs or toxins as well as their metabolites within the whole body, using DESI-MSI.

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Chapter Four – Conclusion & Future Research

Although DESI is a relatively new ionization technique, it is emerging as a powerful new player in surface analyses. Since its conception a decade ago, DESI has been applied towards numerous fields including but not limited to, drug design,[1] cancer screening,[2] detection of explosives[3] and illicit substances,[4] document identification[5] and natural product screening.[6] DESI can be applied towards the analysis of almost any surface. Its soft ionization and flexibility with solvent composition allows DESI the capacity to ionize a range of analytes, such as small organic compounds, polymers, carbohydrates, and protiens.[7] Its open-air arrangement allows for manipulation of the sample even during data acquisitions. MS/MS characterization of compounds can be done directly from a tissue or surface sample, with out the need for any laborious extraction or purification techniques. The process is as simple as moving the sample to position the spray tip above a desired area. DESI is a soft, high-throughput, quick analysis technique that requires little to no sample pretreatments, leaving the sample relatively unchanged from its native state. This enables researchers to perform additional experiments or testing on the same sample.

The capabilities of DESI has only been described here in the application towards two of the many important fields, forensics and tissue analysis. DESI has demonstrated its ability to analyze samples composed of two very different materials, one being a latex polymer base, and the other being biological tissue. For the analysis of sexual assault evidence (condoms and lubricants), DESI was able to analyze the samples within seconds, providing information about relative abundances of commonly used condom manufacturing compounds. The unique, reproducible, mass spectra associated with each different condom brand have established the potential for using these condom "fingerprints" to be able to identify unknown condom brands. This information could have the potential to be used as probative evidence in aiding law enforcement in convicting suspected sexual assault perpetrators. These are only preliminary results to demonstrate the potential of this type of screening. Further research needs to be conducted to create a library of condoms, residues, or individual markers that could be

recognized by the court and law enforcement in connecting a suspect to a specific type of crime. Many sexual assault crimes go unreported because of fear or the knowledge that law enforcement is limited in their ability to find and convict these suspects. Many perpetrators are using protection to avoid leaving biological traces (blood, saliva, semen), therefore finding viable evidence to convict them is becoming scarce. This research could hopefully be used and developed in order to bring closure for victims and justice to the criminals.

The most unique feature of DESI is its ability to transform raw data into beautiful 2 dimensional images capable of displaying a plethora of information. Imaging techniques such as DESI make it possible for researchers to collect a depth of information in such little time compared to previously established techniques. This is because the sample requires little to no pretreatments or lengthy extractions. The imaging of lipids has been studied in the past using rat brains, however whole body analysis of organisms presents a challenge as the time to complete these scans can become lengthy. Zebra fish are small enough that analysis times are on the average 40-70 minutes depending on size and operational parameters. Zebra fish have been gaining popularity since the 1990's as an alternative to using mice as a model vertebrate organism. They have been used extensively in genetic manipulation experiments that have helped us learn more about our own human biology.

Using a morphologically friendly solvent system composed of ACN:DMF (1:1), it has been possible to create ion intensity images of the many organic molecules found within the fish, such as fatty acids, lipids, and bile salts, all while leaving the sample relatively undamaged. By highlighting certain molecules found within the fish, it is possible to identify specific areas, which might be of interest during potential future biological assays with the zebra fish. By selecting a specific lipid, it is possible to identify important parts of the nervous system such as the brain and spinal cord. Some of the lipids identified are even selective towards specific types of tissues within the nervous system such as white and grey matter. The analysis of the zebra fish has also yielded information about the bile salt composition.

Whole body analyses of zebra fish could have potential applications in studying the effects of bioaccumulation of toxic chemicals ending up in rivers and bodies of water. Humans have a huge impact on changing the environment and climate through our industrial and agricultural ventures. Many of the harmful chemicals used enter our water supply through run off or during transportation, which can lead to the accumulation of these toxins within our food supply. It is of utmost importance that humans are aware of the dangers they are introducing to the environment and how it can affect all of us down the line.

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Appendix A – Chapter Two Supplementary Figures

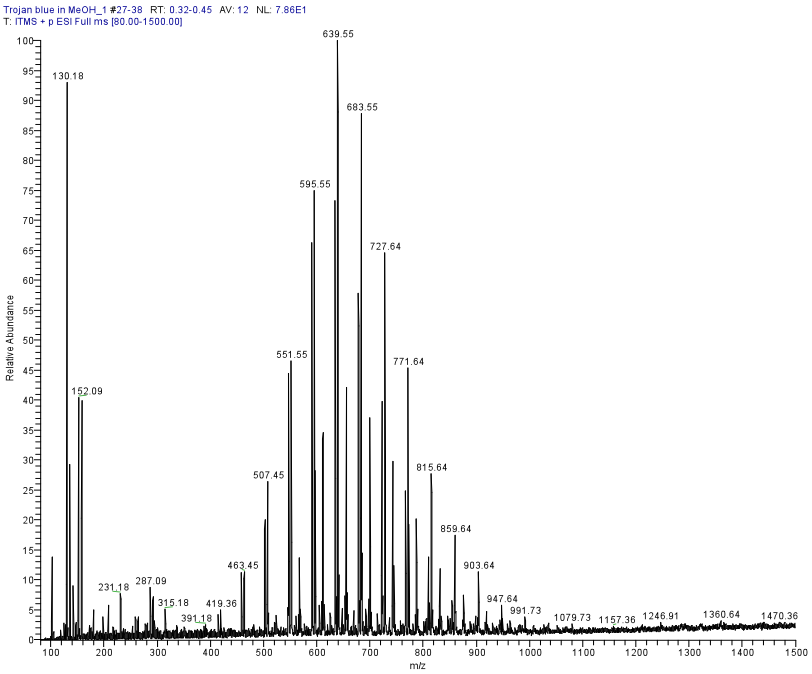

Figure A1 – Sample mass spectrum of a Trojan Enz® condom. Low end *m/z* represents some of the manufacturing compounds, mid-spectrum contains information regarding the polymers added to condom.

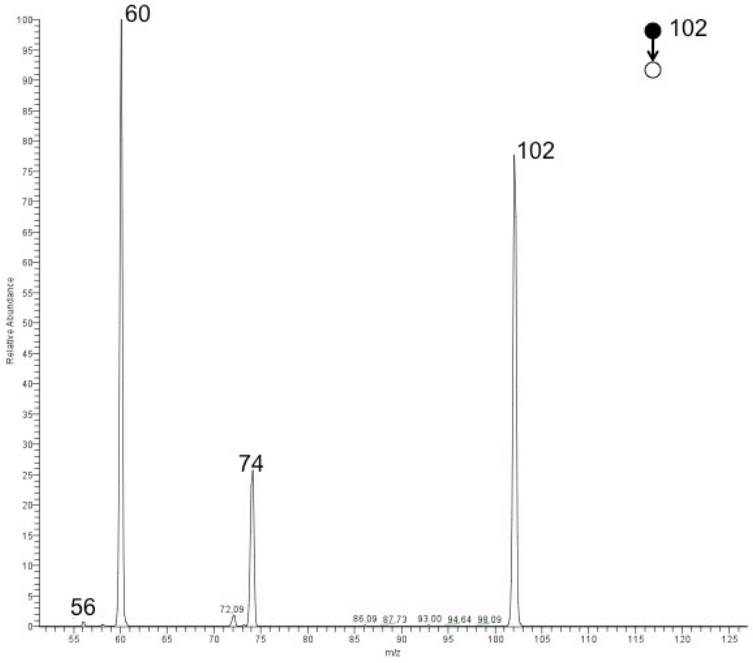

Figure A2 – MS/MS spectrum of N-methylmorpholine (*m/z* 102).

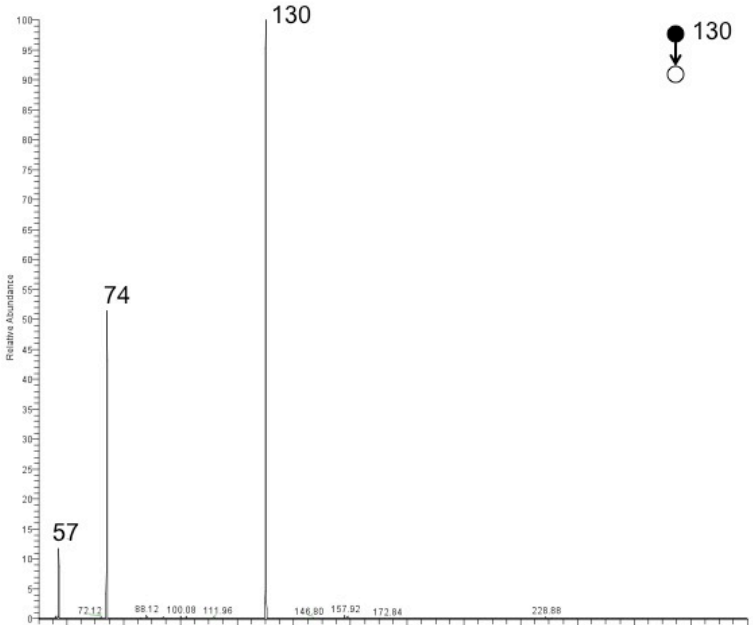

Figure A3 – MS/MS spectrum of N-octylamine (*m/z* 130).

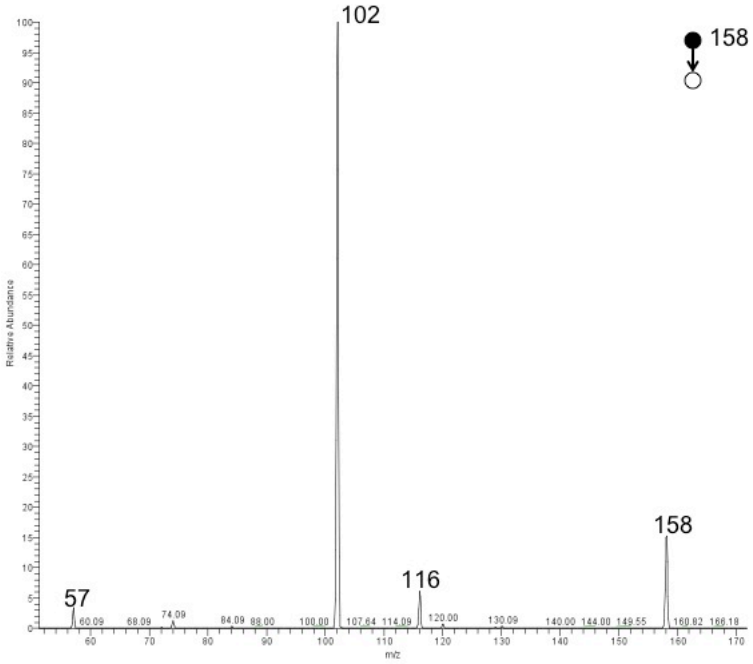

Figure A4 – MS/MS spectrum of N,N-dibutylformamide (*m/z* 158).

Appendix B – Chapter Three Supplementary Figures

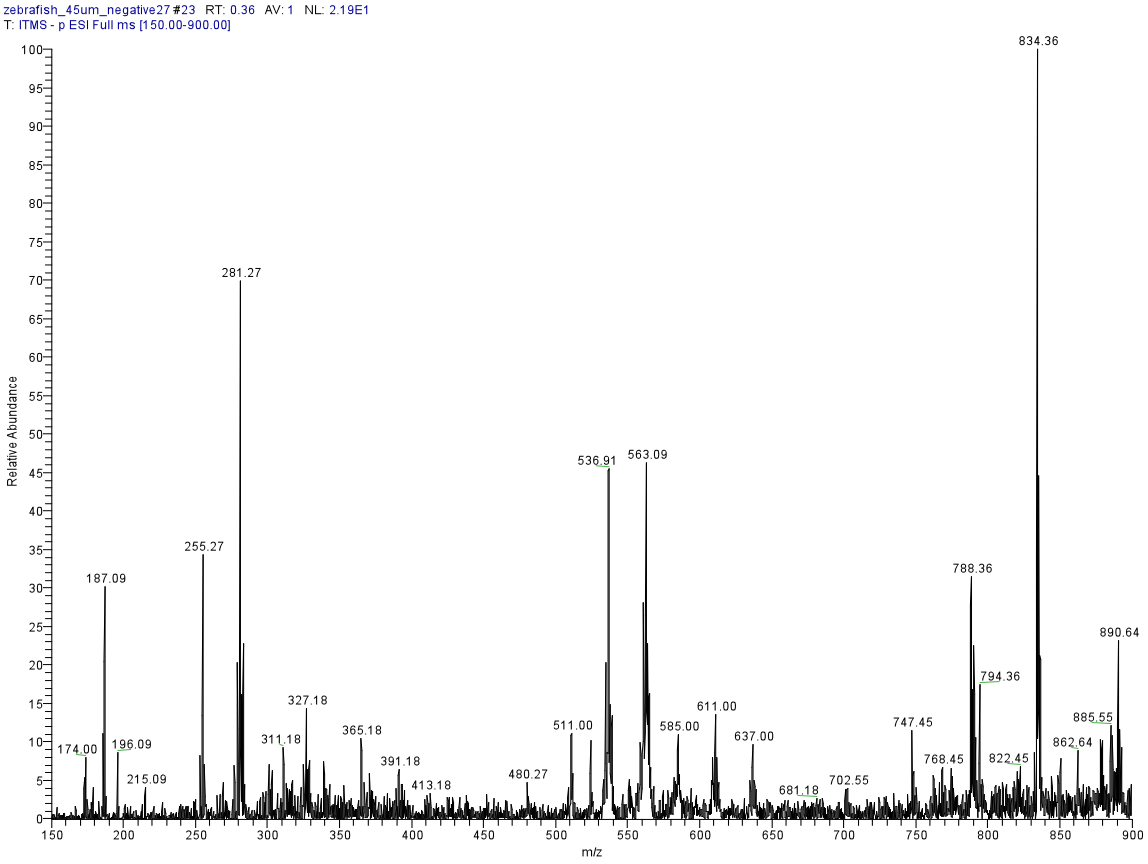

Figure B1 – MS spectrum of a 45µm thick slice of zebra fish analyzed in negative mode using a solvent of DMF:ACN (1:1). This spectrum shows the presence of fatty acids (low *m/z*), their dimers (mid *m/z*), as well as various lipids (high *m/z*).