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Direct Extraction Spray Analysis of Lipid Biomarkers in Injury Rat Spinal Cord

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DIRECT EXTRACTION SPRAY ANALYSIS OF LIPID BIOMARKERS IN INJURY RAT SPINAL CORD

by

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Dedicated to

My Parents, Professors and my friends

who have helped me throughout my life

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ABSTRACT

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Lipids are crucial components of our bodies, not only as the building component of the cell membrane, but also as signaling molecules and an energy storing unit. The study of lipids may bring great improvement for life science. Moreover, lipids may also be a key factor in many diseases, including cancer, Alzheimer's disease, and neural diseases.[1] Qualitative and quantitative lipid analysis of infected tissue is very important when uncovering the cause and mechanism of disease, and can therefore bring great success for the development of diagnosis and treatment of disease.

In this research, we used a photochemical reaction to help determine the double bond location in unsaturated lipids. And by tracking the changes of unsaturated lipid biomarkers in rat spinal cord, we were able to diagnose and monitor the trend of the disease. Mass spectrometry is one of the most powerful analytical instruments for lipid analysis, due to its great sensitivity and specificity. The development of ambient mass spectrometry provides a much more efficient way for chemistry analysis: by optimizing the preparation time and work in sample pre-separation. By coupling Paternò-Büchi reaction with ambient direct extraction spray mass spectrometry, a fast and effective method has been developed to identify and study the biomarkers for spinal cord injury. A couple of fatty acid and phospholipid biomarkers have been determined from the research by comparing the change of lipids between healthy and injured spinal cord rat samples, and the trend of the change of the injury has been monitored and studied. This study provides

one possible method for the diagnosis of spinal cord disease, which may inspire future study in pathology.

1. INTRODUCTION

1.1 Definition and Categorization of Lipids

Lipids are essential component in living organisms, due to their specific functionality and unique structure. The most important role for lipids is the role they play in the cellular membrane. Moreover, lipids can be very significant in the process of energy production, energy storage, hormone production, cellular signaling and so much more.[2–4] With all of the important roles that lipids play in the functioning of living organisms, the study of lipids can be a crucial key to the understanding of many unsolved problems in life science.

According to the definition by Nature magazine, lipidomics is the "study of the structure and function of the complete set of lipids produced in a given cell or organism as well as their interactions with other lipids, proteins and metabolites".[5] Due to lipids' significant role in cell metabolism as well as their other important functions, the study of lipidomics is crucial to the understanding of the biological characteristic and mechanism for living species. The study of lipidomics has been making advances in recent years due to the realization of the crucial role lipids play in multiple diseases.[4] Since lipidomic research requires the identification and quantitative analysis of numerous lipid species, analytical instruments including mass spectrometer, nuclear magnetic resonance and many others are playing a more and more important role in lipidomic research.

The author of *Principles of Biochemistry* Voet defines lipids as "…substances of biological origin that are soluble in organic solvents such as chloroform and methanol".[6] This is the most popular definition for lipids in most textbooks because of its simplicity and clearness. However, the interpretation may not be very precise, since many molecules with very similar characteristics will fall under this definition. Thus, it can be very important to come up with a

better definition of lipids. According to Brown and Murphy, lipids are “Hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensation of the thioesters such as fatty acids or polyketides and/or by carbocation-based condensations of isoprene units such as prenols and sterols”.[7] From the definition, we can clearly see that it is really difficult to categorize lipids under one umbrella term, since different lipids may possess very distinctive characteristics. Hence, rather than using the general term, it can be very helpful to break down the definition of lipids into different sub-categories based on different features.

There are multiple ways to categorize lipids. Generally, they can be classified into non-polar lipids and polar lipids based on the polarity of the carboxylic head group structure.[8]

Nonpolar lipids include cholesterol, cholesteryl ester as well as TAGs, and these lipids mostly differ by the structure of their backbone, which makes it possible to be analyze with NMR shifts.[9] Polar lipids mostly consist of phospholipids, sphingolipids and glycolipids.[9] Phospholipids, as one of the well-studied polar lipids, can line up into lipid bilayers, which is the major component of cell membrane, and they are consist of two hydrophobic fatty acid chains as well as a phosphate head group. Phospholipids can be then classified into phosphatidylcholine(PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PtdGro), phosphatidylinoritol (PtdIns), phosphatidylserine (PtdSer) and phosphatidic acid (PtdH).[8]

Another major type of classification of lipids is based on structural differences, especially the differences for each of its functional groups. Lipids were divided into eight different sub-categories: Fatty acid (FA), Glycerolipids (GL), Glycerophospholipids (GP), Sphingolipids (SP), Sterol lipids (ST), Prenol lipids (PR), Saccharolipids (SL), Polyketides (PK).[10]

Fatty acids are carboxylic acids that contain a carboxyl end group and a hydrocarbon chain, which is an essential component of human diet. Since it can also be the product from the decomposition of phospholipid and glycerides, this characteristic for lipids may be very important for lipidomic analysis.

The classification of fatty acids can be made according to their distinctive functions in two categories: saturated and unsaturated lipids. For unsaturated fatty acids, the position as well as the number of the double bond will also be a factor, which can be divided into monounsaturated fatty acid and polyunsaturated fatty acid. Based on the location of the double bond, fatty acids can be further categorized into w-3 and w-6 type, and so on.

The categorization of lipids provides a distinct picture for the whole lipid map, which greatly reduces the complexity of lipids, making the study of lipidomics more elaborate and more accurate.

1.2 Techniques for Lipid Separation and Analysis

Due to the great variety and complexity of lipids, there has always been a challenge in the analysis of lipids, and the two factors may be even more complicated for lipid analysis in biological tissue samples, as analysis may require extraction from the background interference.

Multiple analytical methods have been used for the analysis of lipids, including thin-layer chromatography(TLC), nuclear magnetic resonance (NMR), and chromatography methods including gas chromatography (GC) and liquid chromatography (LC), as well as mass spectrometry (MS), while GC and LC are commonly combined with MS (GC-MS, LC-MS).[11]

Compared with other analytical methods, mass spectrometry is generally known as the most sensitive and most specific analytical instrument.[12]

However, due to the complex nature of lipids, especially the great variety of lipid isomers, it may be very difficult to solely separate the target lipid isomers for further analysis. Tandem mass spectrometry can be used to determine the structure for the product ion peak after collision induced dissociation, which may be a great supplement to distinguish lipid isomers. But matrix effects may cause great interference in the isolation of target ions.[13] Hence, other instruments are being combined with mass spectrometry to improve the resolution of isomers.

1.2.1 GC-MS and LC-MS

GC-MS combines the ability of gas chromatography and mass spectrometry to separate and extract target compounds for mass analysis.[14] Since GC is mostly used to separate compounds that can vaporize without decomposition, GC-MS can only be used for volatile compounds and compounds that are stable under heating conditions. Thus, GC-MS has been limited to only a few categories of lipids. During the separation, derivatization reactions may be performed on the polar function group, such as carboxylic group, in order to make polar lipid molecules more volatile. Ester derivatization is the most common derivatization methods, using reagents such as 2-propyl ester and methyl ester.[14] Comparing with other separation methods such as LC, GC may be relatively complicated due to the derivatization process. Not only it may be more time consuming, but it may introduce more interference as well as lowering the recovery percentage of compounds after separation.

Currently, LC-MS is still one of the most effective and outstanding separation techniques for lipidomics. Compared with GC-MS, LC-MS is compatible for most lipids, while all nine classes of lipids can be easily detected by LC-MS.[15] Moreover, since LC-MS does not require derivatization, it avoids one of the most time dependent steps for GC-MS, making it much more efficient. By comparing the mass range for both chromatography techniques, GC-MS has a mass

range around 1000 daltons, while the mass range for LC-MS can be much higher, which enables the analysis for some high molecular weight lipids such as phospholipids and sphingolipids. There are several widely used LC separation methods: Normal Phase Liquid Chromatography(NPLC), Reverse Phase Liquid Chromatography(RPLC) and hydrophilic interaction chromatography(HILIC).[16,17] HILIC and RPLC are normally compatible with ESI-MS, while NPLC is commonly combined with APCI. HILIC is mostly used for polar lipid species, while NPLC favors nonpolar lipid species.[18] RPLC is more generally compatible than the other two techniques, due to its great efficiency in separating the lipids.[19,20] In addition, by replacing the regular HPLC with UPLC as the separation technique, a higher separation efficiency can be achieved.

1.2.2 NMR and FT-IR

For lipidomic research, NMR spectroscopy can provide the structural information as well as the molecular dynamic information for lipids.[9] Although it hasn't been a widely used technique compared to HPLC and mass spectrometry, it has its unique advantages for quantitative lipidomic research. Unlike with MS, NMR does not fragment the lipid molecule, which enables recovery for further analysis.[9] Besides, the NMR based analytical technique can achieve direct quantitative information and obtain molecular dynamic information.[9]

NMR can also be coupled with other separation methods that purifies samples for analysis. Thin-layer chromatography, flash chromatography as well as liquid chromatography are often used as the separation and purification tools for NMR.[9]

Due to the similarity and subtle differences of the lipid structure, the sensitivity can be limited by the overlapping of signal peaks. The structure isomers for lipids from the same category may be very difficult to separate. Moreover, since NMR mostly requires pure samples

for analysis, the separation and purification steps may be time consuming. However, despite all the disadvantages, NMR can still be a very useful complementary tool for the lipidomic analysis.[9]

Fourier Transform Infrared spectroscopy (FT-IR) can be used to record the infrared spectrum emission or absorption of the target lipid. Because of the good compatibility as well as the simplicity and quickness of FT-IR, the device has been used as a complementary tool for lipid analysis. Although MS is a more sensitive instrument than FT-IR, the later instrument and the organic solvent it uses is cost effective, and the sample preparation can be simpler and more efficient. As a result, many fundamental laboratories may use it for lipid analysis. Lipidomic research using FT-IR has been recognized for the quantitative analysis of phospholipids, polyunsaturated fatty acids, steroids and so on, showing its prominent ability for lipidomic research.[21]

1.2.3 Ambient Mass Spectrometry for Lipid Analysis

Mass spectrometry has been outstanding for lipidomic analysis because of the great sensitivity and capability. However, there are several downsides for the technology, especially the interference caused by matrix effects.[12] Thus, multiple analytical separation methods have been combined with mass spectrometer to suppress the interferences, for instance, LC or GC. However, the combination of separation methods may increase the complexity as well as the cost for research. In 2004, the concept of ambient mass spectrometry was proposed by Dr. Graham R. Cooks, and ever since, more than 100 ambient ionization studies have been published.[22,23]

Ambient mass spectrometry enables the acquisition of mass spectrum to be done in the native environment, omitting the sample pre-separation step, and directly ionizing the sample outside of the mass spectrometer.[22,12] By using ambient ionization mass spectrometry, the

analysis process can be greatly simplified, without negatively affecting results. Multiple ambient ionization methods have been used for the lipidomic research, such as desorption electrospray ionization (DESI), Direct analysis in real time(DART), paper spray, and so on.[22,24,25]

1.3 The study of Lipid Biomarkers

1.3.1 The Definition and Meaning of Biomarkers

Biomarkers are indicators that can show the status for a disease or medical state, which can be measured accurately and reproducibly.[26] Moreover, the biomarkers should be able to eliminate the interference from external factors, such as individual differences between patients.

The concept of biomarkers has great impact for both pathology and point of care studies. Due to the stability and precision of biomarkers, scientists are able to track and determine the development and the stage of the disease by monitoring the changes in biomarkers. A good biomarker can be a reliable criterion for doctors to make medical decisions, and it can be a great tool for a research study.

Biomarkers can be categorized into imaging/non-imaging biomarkers or molecular biomarkers by their characteristics.[26–28] Imaging biomarkers are used in computed tomography, positron emission tomography and magnetic resonance imaging and so on.[28] Molecular biomarkers can be measured in all kinds of bio samples, including but not limited to blood, tissue biopsy, or body fluid.[28] They can be large molecules such as proteins, or small molecules such as peptides or lipids.[28,29] Biomarkers can also be classified by their applications, such as diagnostic biomarkers, disease stage biomarkers, disease monitoring biomarkers, or even biomarkers for medical feedback after drug usage.[29]

1.3.2 Lipid Biomarkers Introduction

Comparing with other types of molecular biomarkers, such as protein biomarkers, lipid biomarkers have a relatively small molecular weight range, which makes them applicable for most mass spectrometers. Currently, the mass range for most of miniaturized mass spectrometers is only in the hundreds. Hence lipid biomarkers have the advantage of working on small instruments, enabling fast, on-site point of care diagnosis to aid treatment.

Due to the abundance of lipids and their significant role in biological system, lipids show great potential in the diagnosis and monitoring of disease.[4,11] The research on identifying the biomarkers for several popular diseases has shown convincing evidence for this.

For Alzheimer's disease, the phosphatidylcholine and phosphatidylethanolamine level is significantly lower than in healthy individuals or patients with other similar brain diseases, and a near stoichiometric relation can be found between the lipid concentration and the severity of the disease.[1,30]

Biomarkers are also considered as the key to cancer early diagnosis. Phosphatidyl-ethanolamine has been found to have higher percentage on the outer membrane for tumor cell, which make it a great lipid biomarker for cancer cell targeting.[31]

1.4 Significance of Polyunsaturated Fatty Acid C=C Isomers

Unsaturated lipids are lipids that contain at least one C=C double bond on the fatty acid chain. The locaton of the double bond in the lipid is a decisive factor for the the determination of many lipid characteristics.

The degree of unsaturation of a lipid is determined by the number of double bonds on the carbon chains. When there is only one double bond, it is referred to as a monounsaturated lipid, while there is more than one double bond, it is referred to as a polyunsaturated lipid.

Common monounsaturated fatty acids, such as oleic acid and palmitoleic acid, have great abundance in human adipose tissue. For polyunsaturated fatty acid, docosahexaenoic acid (DHA) can be found in fish oil and alga oil, and it has the ability to lower the triglyceride and cholesterol concentration in blood vessels, thus preventing cardiovascular diseases.[32] Eicosatetraenoic acid (EPA) has proven to have great effect in preventing heart attack or strokes.[33] These are the benefits that monounsaturated fatty acid cannot accomplish.

However, it has always been a challenge to determine the double bond position using traditional analytical methods. Classically, researchers will use the ozone oxidative cleavage method to break the double bond of the pre-separated lipids, and then identify the fragment pieces using chromatography.[34] This approach, however, may be difficult to apply to polyunsaturated fatty acid or phospholipids, due to the formation of numerous fragments as well as the challenge for distinguishing the double bond location in isomers.[35] With the development of mass spectrometry, chromatography coupling with mass spectrometry has been the most common used analytical method for lipid analysis. Multiple research approaches have been published, but it has still been a challenge to achieve an online, simple and efficient method to determine the double bond position of unsaturated lipids.[36]

1.5 Paternò-Büchi (PB) Reaction

Paternò–Büchi reaction is an [2+2] photochemical reaction named after Emanuele Paternò and George Büchi.[37–39] The reaction has been widely used in organic synthesis to create a four-member oxetane ring.

The mechanism of the reaction is shown in Figure 1-1. The reaction between the carbonyl group and C=C double bond will create a four-member ring. The intermediate product can easily go through a retro P-B reaction when heated or exposed to UV light, cleaving the ring structure

into an olefin as well as a ketone product. There are two possible pathways for the retro P-B reaction. The first one is returning back to the original reactant product, while the second one is to cleave the original C-O bond at the original C=O double bond position, as well as the C-C bond at the original C=C double bond position. Also, due to the geometry of the reactant in P-B reaction, there may be two possible oxetane ring stereoisomer products formed after the reaction, which is shown in the mechanism.[37] The greatest advantage of Paternò–Büchi reaction is its convenience. The only reaction condition is light, so the whole experiment can be designed into an online device.

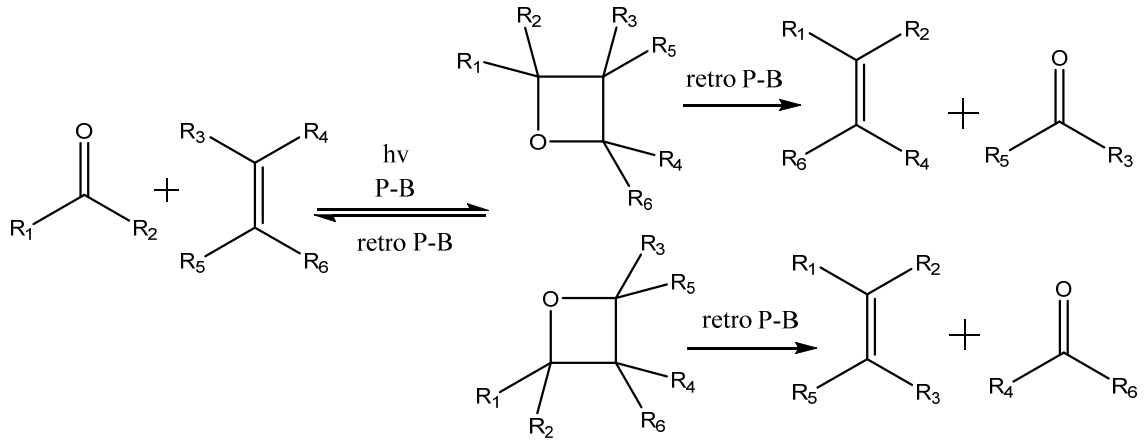

Figure 1-1 Mechanism for P-B reaction

2. DIRECT EXTRACTION SPRAY ANALYSIS OF LIPID BIOMARKERS IN INJURY RAT SPINAL CORD

2.1 Introduction

Spinal cord injury is an injury of the spine that is caused by either contusion or bruising. Due to the inefficiency in endogenous repair of the human central nerve system, the patient can hardly recover from the injury by themselves.[6] Moreover, since the injury mostly occurs at a young age, the cost of the lifelong medical care and treatment is a significant burden to most families as well as the public health system.[6] A spinal cord injury is very common, and since a huge percentage of patients were injured during sporting events, it is a great threat to the health and wellbeing for kids and youth. According to recent data, 2.5 million people were living with a spinal cord injury, and more than 130,000 new injuries are reported annually.[40,41]

There are two stages of Spinal Cord Injury. The first stage, which is also known as the primary stage, is mostly the mechanical destruction of the tissue, causing a series of further damage events in the nervous system, immune system as well as the vascular system.[42] The injury can also cause permanent loss of strength, sensation and other body functions below the location of the injury.[42]

The secondary stage of the injury is a continuation of the initial stage which is mostly based on the damage of the vascular and biochemical organization.[40] A series of biological events such as inflammation, free radical generation will occur during the secondary stage, eventually causing apoptosis and necrosis of the cell.[43,44] Immune response also occurs during the secondary stage of the injury, causing edema, necrotic cell death, as well as the production of reactive oxygen species.[40,42] Since the primary stage of the spinal cord injury happens abruptly, and usually doesn't

last for a long period of time (ranging from a few minutes to several hours), most of the research that has been conducted was focused on the secondary stage of the injury.

However, even though huge resources have been investigated into the research of spinal cord injury, no significant progress in neural recovery or physical rehabilitation has been accomplished. Researchers have put huge efforts on studying the mechanism of the injury, hoping that this will inspire the development of medical treatment. The key idea of the treatment is to stablize the spine, and to control the inflammation, so understanding the mechanism for the injury is crucial. Throughout the years, approximately 25 mechanisms for the secondary stage of the spinal cord injury have been published, but a consensus has yet to be made.[40,45,46]

Most analytical methods that were used in previous research were ex-vivo method, thus it can be hard to monitor the changes and the progression of the disease.[44,47] Additionally, most of the analytical methods need extra preliminary extraction steps.[47] Although the process may significantly improve the quality and purity of the experimental results, the preparation process may be very time consuming, and may be very inconvenient when conducting analysis on a larger scale. Moreover, the extraction method may also possibly introduce external contamination to the sample. Therefore, from all the disadvantages discussed above, the development of a less invasive, in-vivo analysis enabled, fast and convenient analytical technology is a great need.

By using online mass spectrometry analysis on spinal cord research, it became easier and much more convenient to track the changes of the biomarkers, which enable us to study the stage and development of the inflammation.

The mass spectrometer, as one of the most efficient and convenient analytical instrument, has long been used for the study of spinal cord injury.[47,48] The great sensitivity, high selectivity of

mass spectrometer makes mass spectrometry a great analysis method to do both quantitative and qualitative analysis for the lipid in spinal cord injury samples.

Due to the complexity of live animal tissue samples, the matrix effect can be very significant during analysis. For previous research, liquid chromatography (LC) was often combined with mass spectrometer.[47] Multiple preparation steps were conducted to the sample prior entering the LC-MS. However, when large batch of samples are being analyzed, the time and work required for the experiment can be enormous.

To optimize the preparation and separation method, ambient mass spectrometry has been proposed in the last decade. The new method enables the recording of mass spectra on ordinary samples, in their native environment, without sample preparation or pre-separation by creating ions outside the instrument.[22] Multiple ambient ionization methods were proposed during the last few years for spinal cord injury study, including desorption electrospray ionization (DESI), direct analysis in real time, paper spray ionization, etc. All these methods provide a more efficient option for us to analyze complicated biology samples and still be able to get data with good quality. In this experiment, direct extraction electrospray mass spectrometry, which is better known as probe electrospray ionization mass spectrometry (PESI-MS), was used as the analytical method.[23,49,50]

Lipids consist of almost fifty percent of the spinal cord dry weight.[48] When the spinal cord is injured, especially in the secondary stage, lipid oxidation and lipid peroxidation will occur, and reactive oxygen species (ROS) will be generated.[51] The ROS will play a significant role in cell apoptosis as well as necrosis. Multiple lipid species may be involved in the process, and when comparing the healthy spinal cord sample with the injured sample, the changes in both the quantity and the variety of lipids makes it an ideal model for biomarkers.

2.2 Materials and Method

2.2.1 Animal Samples

In this experiment, all animal study protocols have been approved by the Institutional Animal Care and Use Committee (IACUC). 6 Male Sprague-Dawley rats have been used for experiment, provided by Dr. Riyi Shi's research lab from the College of Veterinary Medicine from Purdue University.

2.2.2 Spinal Cord Tissue Preparation

All six rats were anesthetized by injection with ketamine and xylazine mixture. Three of them are spinal cord injured rats, marked as a disease sample, while the other three healthy rats were marked as a control healthy sample. The three disease rats were injured at the center of their spinal cord. When the animal was fully anesthetized, the spinal cord injury contusion was produced by an impactor, by dropping a 10g rod from a height of 37.5mm onto the center of the spinal cord, marked as epicenter. All six rats were sacrificed 24 hours after the spinal cord injury. The spinal cords were then extracted, and cut into 1cm pieces. Each spinal cord piece was labeled for their position on the spinal cord, such as up 1cm, epicenter, down 1cm, etc. The scheme is shown in Figure 2-1.

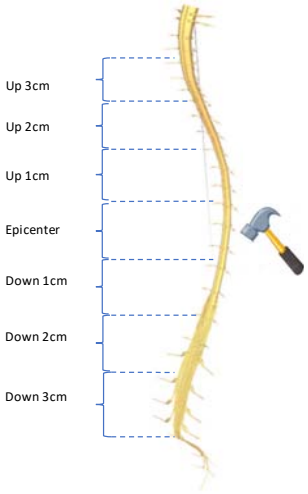

Figure 2-1 Spinal Cord Injury sample preparation

2.2.3 Instrument

All MS experiments were performed on a 4000 QTRAP triple quadrupole/linear ion trap hybrid mass spectrometer (Sciex). The instrument was set as follows: curtain gas, 5 psi; declustering potential, 20V, and the use of Q3 as linear ion trap. Settings may vary due to different conditions of the instrument.

2.2.4 Direct Spray Analysis

The experiment set up is shown in Figure 2-2. A UV lamp (wavelength at 254nm) was put right next to the nanoESI tip in order to perform an online reaction. A steel wire was used to penetrate the tissue sample three to four times, in order to get as much tissue attached to it. Then the stainless-steel wire will be inserted in to the nanoESI tip, preloaded with 20μL of a mixture of acetone, acetone nitrile and water (70/20/10, v/v/v), and 1%(v) ammonium hydroxide was added for negative ion mode detection (1%(v) formic acid for positive ion mode detection). The nanoESI tip was attached to a power source, and an electrospray ionization was performed. After the product ion peak was observed, the UV lamp was turned on to initiate the reaction. Due to

large tissue extraction size during the direct analysis, the nanoESI tip can easily get clogged by the fragments of the tissue after 10 seconds. Since the reaction is performed online, the spray needs to be consistent for at least 30 seconds. Thus, a larger size tip has been made to adapt to the analysis of live samples. The larger the tip, the higher the voltage is applied onto the nano tip; so, there may be a tradeoff between the signal intensity and the consistency of the spray.

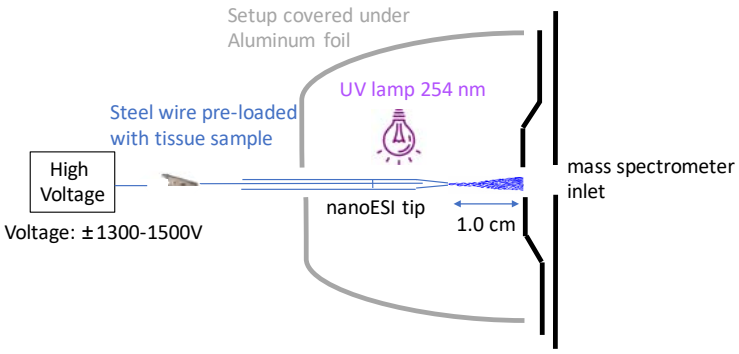

Figure 2-2 Instrumentation setup

2.3 Results and Discussion

2.3.1 Identification of Lipid C=C Location Isomers with PB-MS/MS

As mentioned in the introduction section, with lipids playing essential biochemical roles in living organisms, the species and quantity of lipids may vary between healthy and injured tissue samples. Because of the unique characteristic of Paternò-Büchi reaction, we are mainly focusing on the difference in the double bond position in lipids between healthy and injured tissues. By comparing the mass spectrum for disease tissue samples and healthy tissue samples, we were able to determine biomarkers that can show evidence for the existence of the disease. In this experiment, we were trying to analyze the correlation of double bond location on the lipid chain between spinal cord injured samples and healthy samples.

P-B reaction may have multiple benefits for the identification and analysis of biomarkers in tissue sample. First of all, the addition of a mass corresponding to a ketone can be seen on the mass spectrum after the P-B reaction. In this experiment, an addition of a 58 daltons molecule from the original unsaturated fatty acid can be observed, indicating the addition of an acetone to the structure. Moreover, after taking the MS/MS spectrum of the P-B reaction product peak, the collision induced dissociation ion fragment can help indicate the double bond position on the original lipid.

According to Figure 2-3 A, the lipid molecule that we focused on is fatty acid 18:1. In tissue sample, the FA 18:1 is usually consist of a mixture of Δ9 and Δ11, which indicates that the double bond is on the 9^{th}-10^{th} carbon and the 11^{th}-12^{th} carbon. A m/z 339.3 peak can be seen after the photochemical reaction, showing it is an +58 Da peak from the fatty acid 18:1 peak at m/z 281.3.

By taking the MS/MS spectrum of the m/z 339.3 product ion, a subsequent CID fragment peak was observed, shown in Figure 2-3 A. Other than the original fatty acid fragment peak at m/z 281.3, which is formed by retro P-B reaction, two pairs of diagnostic ions at m/z 171/197 and m/z 199/215 can be detected. Since there are two location isomers present for the fatty acid at m/z 281.3, the combination for the P-B reaction may create a total of four isomers. Each isomer collapsed into a diagnostic ion peak, thus forming altogether two pairs of ions, one from each fatty acid isomer. These two pair of diagnostic ions peaks determined the location of the double bond on the fatty acid chain, proving that the double bond position is at Δ9 and Δ11. The mass difference between the two pairs of ions are both 26 daltons, which is the mass difference between $-C(CH_3)_2$ and =O, confirming the structure shown in the mechanism Figure 2-3 B. The

ratio between the 171/199 and the 197/215 peak are consistent, showing that the ratio between the Δ9 isomer and Δ11 isomer is about 1:3.

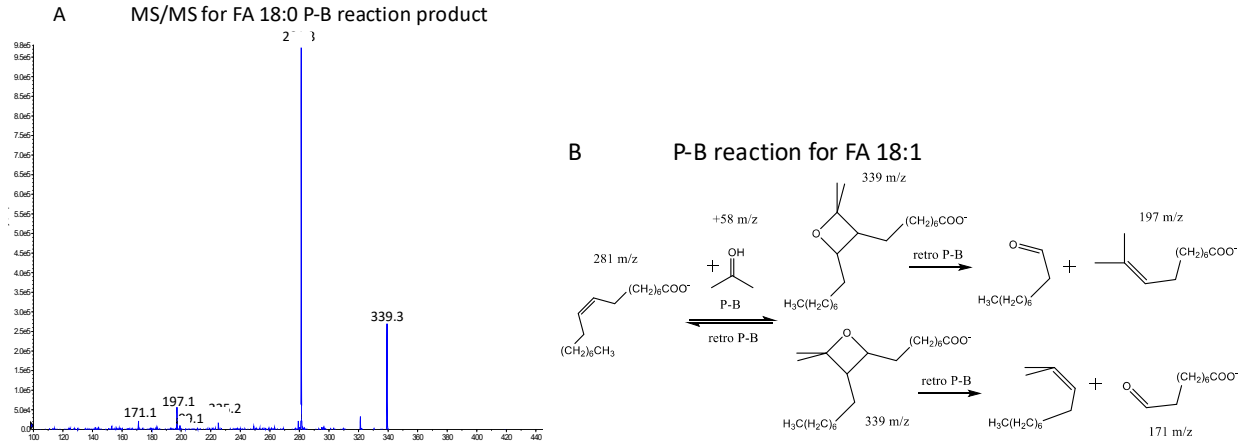

Figure 2-3 A) MS/MS of the P-B reaction product for FA 18:1 (m/z 339.3) B) Schematic representation for the mechanism for FA 18:1 P-B reaction and the mechanism for the fragmentation of P-B reaction product

2.3.2 Fatty Acid Biomarker Identification in Healthy and Spinal Cord Injury Tissue Samples

2.3.2.1 Full Lipid Profiling

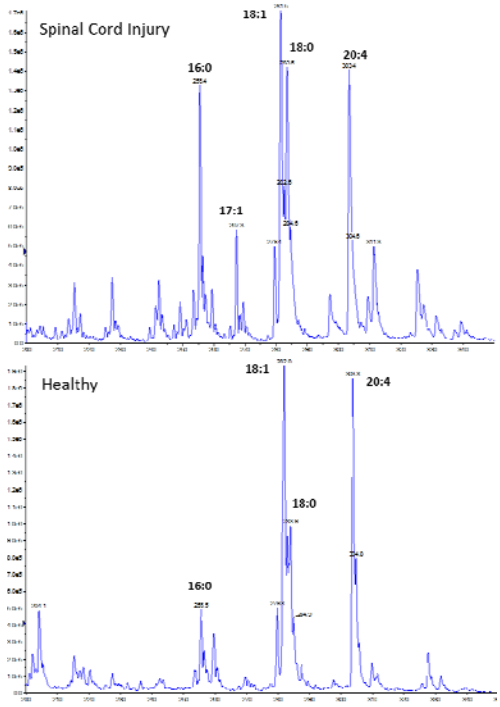

Figure 2-4 Full lipid profiling comparison between spinal cord injury sample and healthy sample before P-B reaction

By comparing the full lipid profiling spectrum between the spinal cord injured samples and the healthy samples, several differences can be found, shown in Figure 2-4. The abundance of fatty acids may vary between different rats, but while comparing the general distribution of the lipids, we were able to see that FA 16:0 (m/z 255), FA 18:0 (m/z 283), FA 18:1(m/z 281), FA 18:2(m/z 279), FA 20:4 (m/z 303) are the most abundant lipid peaks in both healthy rat and injured rat. The intensity for FA 16:0 at m/z 255 is relatively higher in the spinal cord injured tissue, compared with the healthy samples. FA 17:0 is another significant difference between injured samples and healthy samples. The peak at m/z 267 can rarely be observed in the healthy

tissue samples, while in the spinal cord injured samples, it is a distinct peak. Other fatty acids neither have good intensity in spinal cord samples, nor show consistent differences between the two types of samples, thus they are not considered as reliable biomarkers.

Since we did not introduce internal standard to the experiment, it is difficult to accomplish quantitative analysis for the lipid full profiling. An ideal internal standard for this experiment should be another C=C location isomer that does not exist in the sample, thus making it possible to sketch a calibration curve for accurate quantitative analysis. However, due to the complexity of live tissues, it can be very complicated to find an internal standard. For future research, it is possible that we can try to synthesis some deuterated isomers for better quantitative analysis.

2.3.2.2 Fatty Acid Analysis

We were not able to quantitatively compare the intensity of the peak between the spinal cord injured samples and the healthy samples, since no internal standard was introduced. However, the ratio between the isomer peaks should always be relatively consistent with similar species. By monitoring the differences between spinal cord injured samples and control samples, the ratio varies between the two species.

For polyunsaturated fatty acids, the location of the double bond can have distinctive difference between healthy and injured samples. Fatty acid 18:1, as shown in the previous experiment, has two major stereoisomers, Δ9 and Δ11, depicted in Figure 2-5. By comparing the ratio between the two isomers Δ9/Δ11, shown in table 2-1, the injured spinal cord samples have a relatively higher ratio than healthy samples. The mean of the Δ9/Δ11 ratio for the three injured rats is 2.69±0.15, while the mean for the three healthy rats is 1.93±0.17. The difference between the ratio is significant, showing its potential to be a convincing biomarker. However, the sample

size may be too small due to the lack of injured tissue samples, thus for future clinical research, large batch experiments should be conducted.

In lipid samples, other fatty acid may also possibly have stereoisomers that have ratio difference between healthy and injured samples. But most of the lipids in tissue samples do not have good intensities, which may result in poor MS/MS fragment peak intensities. Thus, the ratio for FA 18:1 stereoisomers is our main interest.

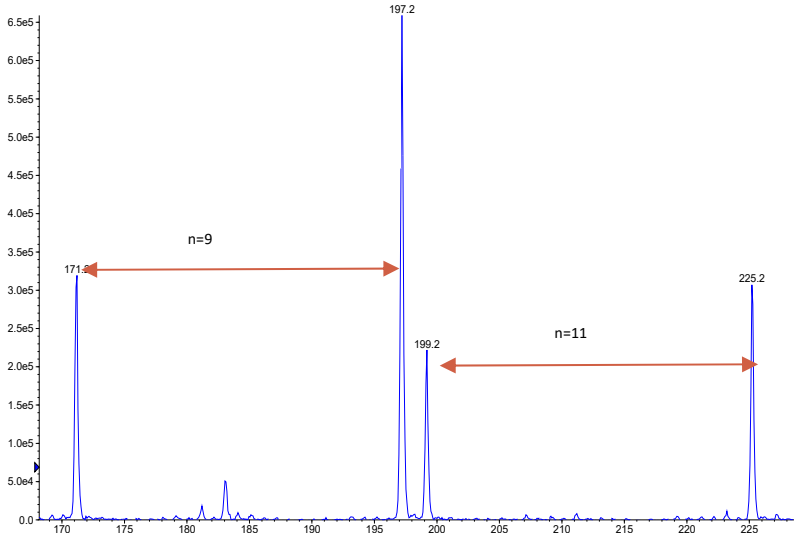

Figure 2-5 Δ9 and Δ11 stereoisomers from the fragments of P-B reaction product for FA 18:1

Table 2.1 Comparison of the Δ9/Δ11 ratio for FA 18:1 between healthy and spinal cord injured tissue samples

FA 18:1 Δ9/Δ11	Rat 1	Rat 2	Rat 3	Average	STD
Spinal Cord Injured	2.86	2.58	2.63	2.69	0.15
Healthy	1.87	1.80	2.13	1.93	0.17

2.3.3 Phospholipid Biomarker Identification in Healthy and Spinal Cord Injury Tissue Samples

Phospholipids have a structure consisting of two fatty acid tail chains, and connected by a phosphate head group, which makes the analysis similar to the study of fatty acid. Moreover, due to the existence of multiple fatty acid chains, there may be multiple double bonds on each lipid branches, creating an even complicated case for analysis. Therefore, we mainly focused on phospholipids that has an 18:1 chain and on the ratio between the two stereoisomers Δ9 and Δ11. Phosphatidylcholines(PC) has been known as one of the most abundant species in phospholipids in mammals, thus we choose PC 16:0-18:1, PC 18:0-18:1, PC 18:1-18:1 for our main interest.

For PC 16:0-18:1, we were able to see an m/z 818.3 peak show up in the full spectrum after the reaction. This indicates the addition of the acetone (m/z 58) to the PC 16:0-18:1 (m/z 760.3). After taking the MS/MS spectrum for the m/z 339.3 product ion, we were able to compare the ratios Δ9/Δ11. According to the table 2-2, the average ratio for injured samples is 2.77±0.29, while it is 2.25±0.29 for healthy tissue samples. For PC 18:0-18:1, an m/z 846.3 peak can be recorded after the P-B reaction, which indicates the addition of the acetone (m/z 58) to the PC 18:0-18:1 (m/z 788.3). The average ratio for injured samples is 4.18±0.59, and for healthy samples is 3.66±0.14. For PC 18:1-18:1, we were able to see an m/z 844.3 peak shown up after the reaction. This indicates the addition of the acetone (m/z 58) to the PC 18:1-18:1 (m/z 786.3). The average ratio for injured samples is 1.57±0.27, and 1.47±0.12 for healthy samples.

The Δ9/Δ11 ratio for spinal cord injured tissue is clearly larger than healthy tissue samples, showing the potential that Δ9/Δ11 ratio can be a biomarker for diagnosing the injury. However, the standard deviation (STD) is relatively high, due to the limit of sample size. The ratio for rat 2 in the spinal cord injury group deviates from the rest of the data, which may also be a result of the large STD value.

Table 2.2 Comparison of the Δ9/Δ11 ratio for PC 16:0-18:1, PC 18:0-18:1, PC 18:1-18:1 between healthy and spinal cord injured tissue samples

PC 16:0-18:1	Rat 1	Rat 2	Rat 3	Average	STD
Spinal Cord Injured	2.76	2.49	3.08	2.78	0.29
Healthy	2.38	2.45	1.92	2.25	0.29
PC 18:0-18:1	Rat 1	Rat 2	Rat 3	Average	STD
Spinal Cord Injured	4.45	3.50	4.60	4.18	0.60
Healthy	3.80	3.52	3.65	3.66	0.14
PC 18:1-18:1	Rat 1	Rat 2	Rat 3	Average	STD
Spinal Cord Injured	1.56	1.30	1.84	1.57	0.27
Healthy	1.54	1.54	1.33	1.47	0.12

2.3.4 Analysis of the Trend of the Disease Along the Spinal Cord

One of the special features for spinal cord injury is that it typically starts with an abrupt hit on a single spot on the spine, and with the generation and spread of reactive oxygen species, the injury gradually develops into later stages. Different stage of the injury may have different symptoms, and may require different treatment. Therefore, it is important to monitor and analyze the development and the biological state for each stage of the injury.

According to Figure 2-6, by labeling the abundant fatty acid peaks on the full scan spectrum, we were able to see the trend of how lipid changed while moving up from the center of injury spot (epi center). Although quantitation analysis cannot be conduct, the intensity of the biomarker of fatty acid 16:0 (m/z 255) decreases when moving up along the rat spinal cord. The fatty acid 17:1 (m/z 267) also has a tendency to decrease. However, since the intensity for FA 17:1 is relatively low, it is hard to compare the change of the intensity solely from the spectrum. The trend of the lipid abundance decreases when moving further away from the epicenter, which proves that the injury affects the rat spine through the spread of certain substances.

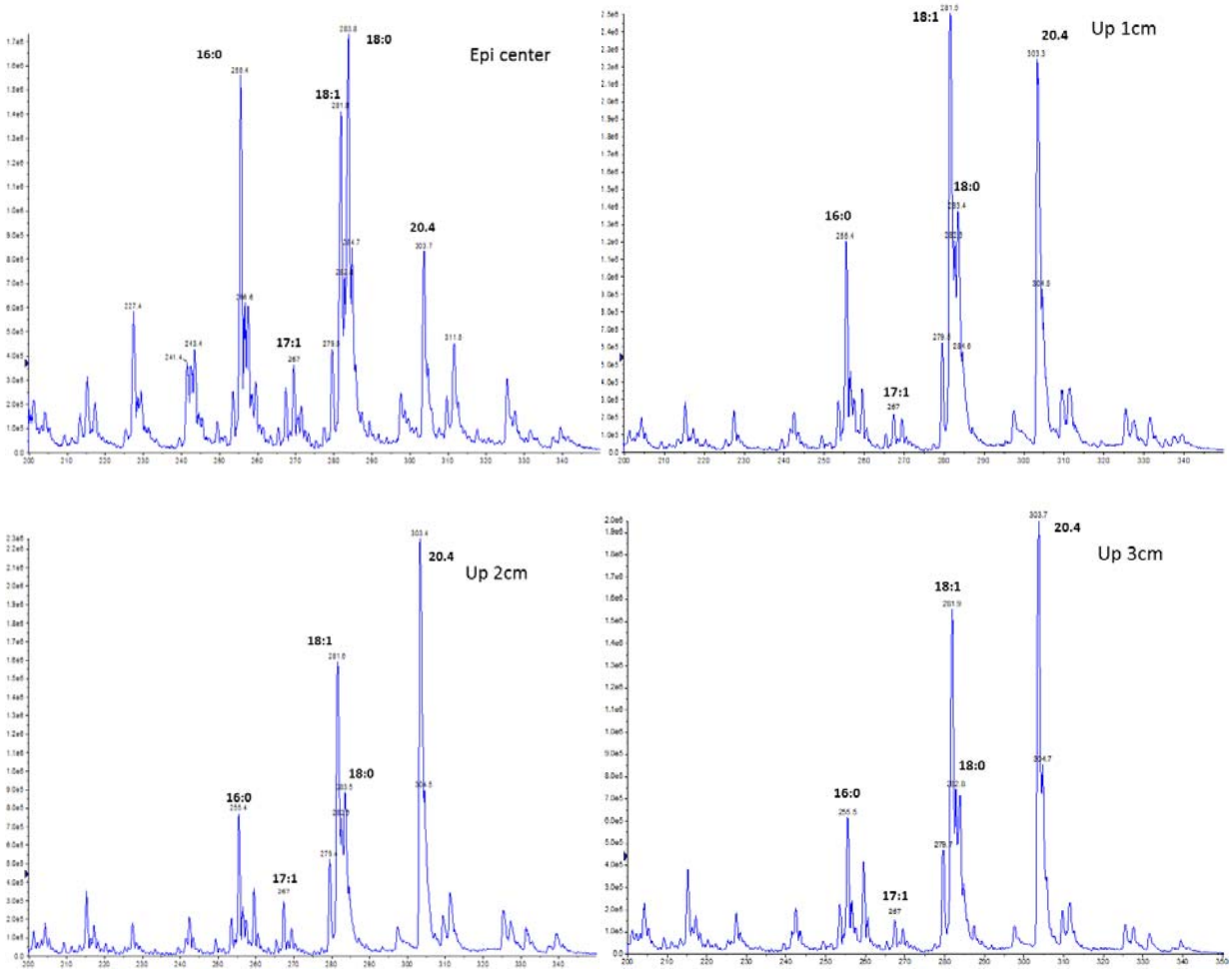

Figure 2-6 Fatty acid profile for spinal cord injured tissue at epicenter, up 1cm, up 2cmand up 3cm

Comparing the Δ9/Δ11 ratio for both FA 18:1 as well as the three phospholipids, shown in Figure 2-7, the graph for fatty acid 18:1 provides the best correlation, showing that the further away from the epi center, the lower the Δ9/Δ11 ratio. For the three phosphatidylcholines, PC 16:0-18:1, PC 18:0-18:1, PC 18:1-18:1, the Figure does not provide strong evidence to show the relationship between the Δ9/Δ11 ratio and the location of the analyte.

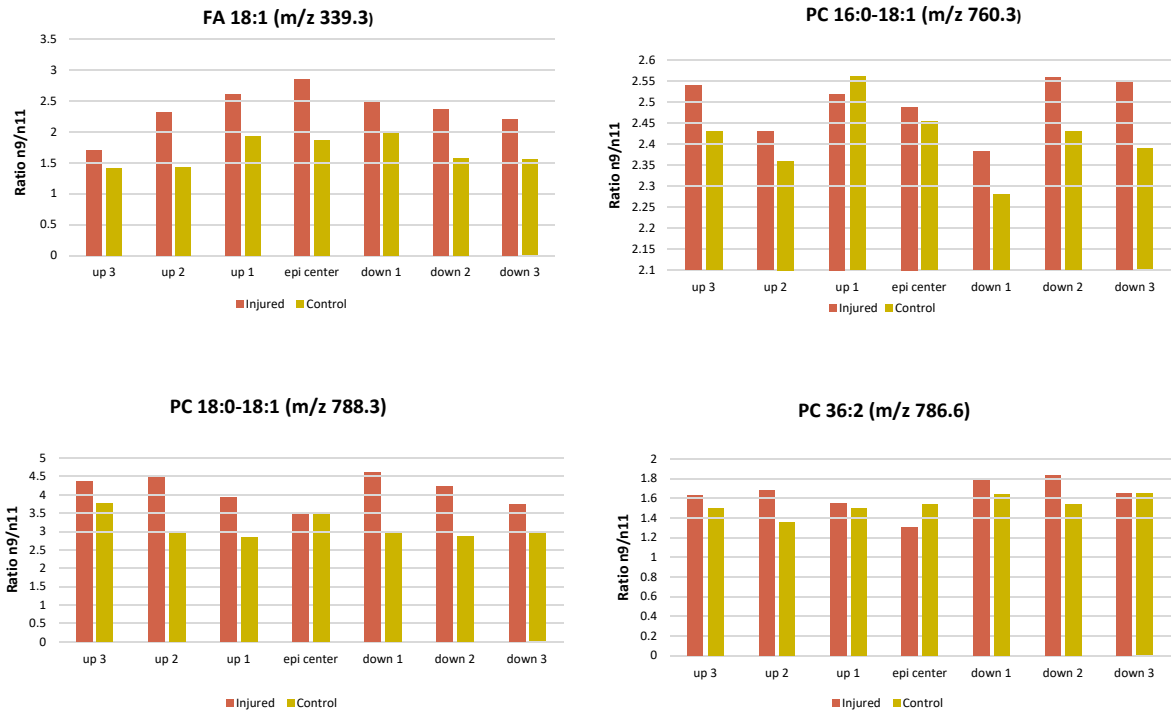

Figure 2-7 Δ9/Δ11 ratio Fatty acid and Phospholipid at different location on the spinal cord

2.3.5 Conclusion

In this study, direct extraction spray analysis provides a fast and efficient way to determine the biomarkers in spinal cord injury tissue samples. By coupling the spray method with online P-B reaction, it is possible for us to determine the double bond location as well as the structure of lipids by taking the MS/MS spectrum for the precursor ion. Lipids were extracted by the use of a steel wire poking into the spinal cord, and ESI was performed for mass analysis. By comparing the healthy and injured rat spinal cord lipids, the abundance of fatty acid 17:1 and fatty acid 16:0 showed a difference in the full lipid profiling scan. The ratio between the Δ9/Δ11 isomers for fatty acid 18:1 increased when the spinal cord is injured, providing a possible biomarker for the diagnosis of the injury. For phospholipids, PC 16:0-18:1, PC 18:0-18:1, PC

18:1-18:1 also show a similar result for the comparison of Δ9/Δ11 ratio. FA 18:1 as well as the three phospholipids show consistency with the location on the spinal cord. The closer it is to the spot of injury, the larger the Δ9/Δ11 ratio is. This may have a great advantage for monitoring the development of spinal cord injury.

For the current methods, there are many places left for improvement. The sample size for the experiment is relatively small, causing a large error bar, thus not very convincing for the conclusion. For further experiments, more parallel tests will be completed in order to increase the accuracy of the result. Moreover, the current method is not able to identify the cis-trans configuration of the double bond. Thus, a modification for the online reaction can be made for better analysis.

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After he graduates, he plans to go back to China for a future career. Jobs in industries, universities and companies are all in consideration for the next stage of his life.