NANO-DESI IMAGING OF EICOSANOIDS IN MOUSE KIDNEY TISSUE USING SELECTED ION MONITORING

by

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ABSTRACT

Nano-DESI mass spectrometry imaging (MSI) is a technique for label-free spatial and molecular characterization of surfaces and biological samples. Eicosanoids are lipid mediators derived from eicosapolyenoic acid- products of arachidonic acid oxidation. Eicosanoids have been of interest to the medical field for many years. Major focus on this lipid class came from the development of nonsteroidal anti-inflammatory drugs (NSAIDs), some of these including aspirin, naproxen, ibuprofen, and acetaminophen work by blocking either the formation or the effects of eicosanoids. These lipids also play important roles in various body functions (cardiovascular, renal, gastrointestinal, neuronal) and as mediators of inflammation, asthma, fever, pain, hypertension, and stroke. Typically, eicosanoids occur in subnanomolar concentrations, despite their high level of bioactivity, which makes them significantly more difficult to analyze via direct mass spectrometry. Here, selected ion monitoring (SIM) is used to increase the signal-to-noise of the identified eicosanoids compared to a standard broadband full scan mode.

CHAPTER 1. INTRODUCTION

Eicosanoids are lipid mediators derived from eicosapolyenoic acid -products of arachidonic acid oxidation. Eicosanoids have been of interest to the medical field for many years. The first main focus on this lipid class came from the development of nonsteroidal anti-inflammatory drugs (NSAIDs), some of these including: aspirin, naproxen, ibuprofen, and acetaminophen work by blocking either the formation or the effects of eicosanoids. These lipids also play important roles in various body functions (cardiovascular, renal, gastrointestinal, neuronal) and as mediators of inflammation, asthma, fever, pain, hypertension, and stroke.[1,2]

The eicosanoid class of lipids includes prostaglandins, prostacyclin, thromboxane, leukotrienes, lipoxins, epoxides, and alcohols (HETEs). Arachidonic acid can be oxidized to at least 30 structurally different primary eicosanoids, each having unique biological origin and properties. Several specific oxidative pathways incorporate oxygen and rearrange the 20-carbon arachidonic acid skeleton, and almost all cells can biosynthesize eicosanoids by at least one mechanism. Cyclooxygenase (COX)-derived eicosanoids have important roles involved in regulation of cardiovascular function and tumor progression. Lipoxygenase (LO)-derived eicosanoids have been implicated as important mediators of inflammation, asthma, cardiovascular disease, and cancer. Cytochrome P-450 (P450)-derived eicosanoids are both vasodilators and vasoconstrictors.[2,3]

Prostaglandins (PGs) are products of the COX pathway of arachidonic acid (AA) metabolism, and PG-glycerol esters (PG-Gs) are products of oxygenation of the endocannabinoid, 2-arachidonoylglycerol (2-AG). The COX product PGH2 is isomerized by prostaglandin synthases to PGE2, PGD2, PGF2α, PGI2, and TxA2. PGs have been implicated in diverse physiological and pathophysiological responses, such as platelet aggregation, gastrointestinal integrity, wound healing, inflammation, hyperalgesia, and fever.[4,5] Similarly, hydroxyeicosatetraenoic acids (HETEs) are products of the LOX and CYP450 pathways of the AA metabolism. In particular, 11-,12-, and 15-HETE are produced by the CYP450 pathway of propylene oxidation, where HpETEs produced by lipoxygenase and cyclooxygenase are reduced by peroxidase.[6]

Typically, eicosanoids occur in subnanomolar concentrations, despite their high level of bioactivity, which makes them significantly more difficult to analyze via direct mass spectrometry.[1] There have been a few different techniques used to increase the signal seen from

these lipids.[1] Prostaglandins are known to form complexes with silver, forming $[PG + Ag]^+$ ions, in which some studies have shown an approximate 30 fold increase in sensitivity.[7] The silver ions form weak charge transfer complexes with alkenes, this characteristic binding results in the addition of Ag^+ to a double bond which can increase ionization efficiency and detectability of a compound.[8] Nano-DESI imaging of $[M + Ag]^+$ species can be realized by adding AgNO3 into the nano-DESI solvent.[7]

Another method of identifying these low concentration lipids is through LC/MS-MS analysis.[3] This has been the most commonly used method of analysis in recent years but does require a more extensive sample preparation than nano-DESI analysis and does not provide localization information. Because PGD2 and PGE2 are geometrical isomers that show similar fragmentation patterns, complete chromatographic separation with excellent peak shape is essential for accurate quantitation.[3]

Previous work has used Selected Ion Monitoring coupled with the nano-DESI source to increase the signal and image low abundance nicotine in mouse brain tissue, using a SIM window of m/z 133-193.[9] SIM mode has also been used with the nano-DESI source to image diclofenac and its low concentration metabolites in mouse kidney and liver tissue.[10]

By using our nano-DESI MSI source[11,12] coupled with a Q-Exactive HF-X Orbitrap Mass Spectrometer mouse kidney tissue was analyzed in standard broadband mode as well as in selected ion monitoring (SIM) mode. The MSI data obtained was analyzed and imaged using the program PeakbyPeak and our own imaging workflow. For peak identifications MS/MS line scans were run on the kidney tissue, and fragmentation spectra matched with the METLIN database.

CHAPTER 2. MASS SPECTROMETRY IMAGING AND NANO-DESI

Mass spectrometry imaging allows for label-free spatial mapping of molecules directly from the surface of tissue, which makes it a powerful analytical technique for applications in biology, clinical research, and drug discovery by providing insight into tissue heterogeneity.[13–17]

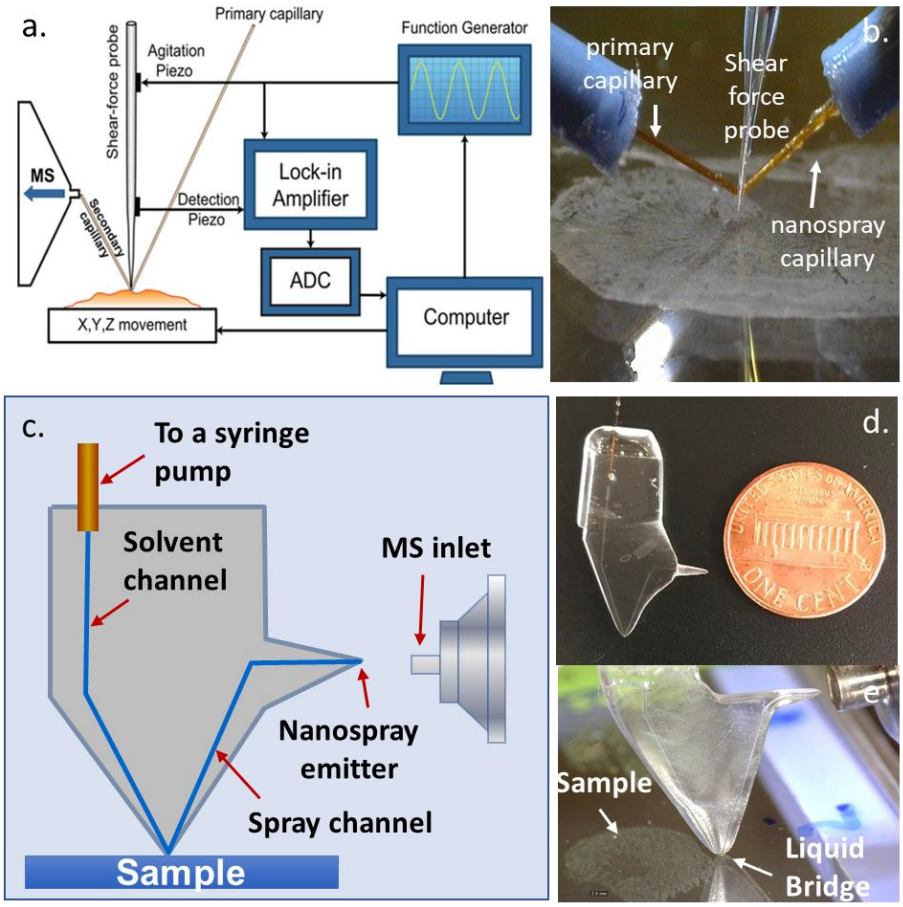

Figure 1: **a.** Schematic drawing of the nano-DESI MSI source. The nano-DESI probe composed of the primary and nanospray capillaries is positioned in front of the mass spectrometer (MS) inlet, and the shear force probe positioned in close proximity to the nano-DESI probe is used to maintain a constant probe-to-sample distance, adapted from reference 11. **b**. A photo of the probes and sample recorded by a side-view microscope during an imaging experiment. **c** Schematic drawing of the iMFP, adapted from reference 12 **d** Photograph of the iMFP, adapted from reference 12. **e** Photograph of the liquid bridge on the glass surface near a mouse kidney tissue sample.

As summarized by our lab's previously published protocol,[18] nano-DESI MSI is an ambient ionization technique based on localized liquid extraction, which enables sensitive and quantitative analysis of molecules from a sample without requiring any sample pretreatment. In nanoDESI MSI,

the spatial resolution is determined by the size of the liquid droplet formed between two glass capillaries, which is controlled by the size of the capillaries, their position, and the flow rate. The capillaries make up a nano-DESI probe, which is positioned at a controlled distance above the sample surface to prevent the probe from either crashing or losing contact with the sample.

This can be done in multiple ways. The first being to control the probe-to-sample distance using a three-point plane mode, in which the vertical position of the sample is automatically adjusted to maintain a constant probe-to-sample distance based on the defined tilt of the flat sample surface. This enables automated imaging of relatively flat tissues with a spatial resolution of 20–100 μm.[18] A second approach has been developed to handle tissue with a rough surface, also allowing for a spatial resolution of better than 10 μm; the distance between the sample and nano-DESI probe can be precisely controlled to within 1 μm by using shear force microscopy (Figure 1a, 1b).[11]

We have also used a newer nano-DESI probe, an integrated microfluidic probe(iMFP), in this work. As summarized in our previously published work,[12] this probe is comprised of a fused silica capillary (50μm ID, 150μm OD) inserted into the glass chip's solvent channel coming down to a finely polished sampling port which is met with the spray channel that transfers the charged solvent now mixed with ions from the tissue surface to the MS inlet (Figure 1c, 1d, 1e). The iMFP is mounted onto a micropositioner and positioned in front of the MS inlet.

CHAPTER 3. METHODS

3.1 Tissue Collection and Handling

Female C57BL/6 mice were sacrificed after 30 minutes by CO2 asphyxiation. All animal experiments were performed according to the institutional guidelines of Merck & Co., Inc., Kenilworth, NJ, USA. Kidney were snap frozen in hexane/dry ice bath and stored in a -80 °C freezer. Tissues were sectioned (thickness = 12 µm) using a Leica CM3050 S cryostat (Leica Biosystems, Buffalo Grove, IL). Sections were thaw-mounted onto glass slides for the imaging experiments, shipped on dry ice, and stored in a -80 °C freezer until the analysis.

3.2 Chemicals

Arachidonic acid-d8 (AA-d8) was purchased from Sigma (St. Louis, MO) was used as an internal standard for the negative mode nano-DESI MSI experiments. Prostaglandin E1 (Cayman Chemical, Ann Arbor, MI) used for suppression comparison with AA-d8. HPLC grade methanol and water for nano-DESI analysis were purchased from Fisher Scientific (Hampton, NH).

3.3 Nano-DESI Imaging

All MSI data collected using custom nano-DESI MSI source[11] with shear force feedback, or our nano-DESI chip setup[12] coupled with a Q-Exactive HF-X Orbitrap MS. A syringe pump (Fusion 100, Chemyx Inc., Stafford, TX) with a 2.5 mL Hamilton syringe was used to deliver a 0.5 µL/min flow of the solvent used; 5µM solution of arachidonic acid-d8 (AAd8) in 9:1 methanol:water. For the standard capillary setup probe is comprised of two 150 µm OD x 50 µm ID fused silica capillaries. The primary capillary delivers the extraction solvent (9:1 MeOH/H2O) to the sample and a secondary capillary transfers extracted molecules to the MS inlet. A third capillary is used as the shear force probe to maintain a constant distance between the nano-DESI probe and the sample[18] The shear force capillary (800 µm OD x 200 µm ID) is pulled to a 20 µm OD tip using a P-2000 micropipette puller system (Sutter Instruments, Novato, CA). All capillaries are positioned using high-resolution micromanipulators (XYZ 500MIM, Quater Research and

Development, Bend, OR) and monitored using two Dino-Lite digital microscopes (AnMo Electronics Corporation, Sanchong, New Taipei, Taiwan).

The spacing between the lines was 100 µm setting the upper limit of the spatial resolution to 100 µm. The spray head was positioned ~1 mm from the tissue surface at a 70° angle to generate a < 0.1 mm spray spot on the substrate. A spray voltage of 3.2kV was applied to the solvent line. The capillary inlet temperature was set at 250°C. For the capillary setup, the scan rate was 35µm/s with an image pixel resolution of approximately 100 micron and a 2.66 Hz data acquisition rate for SIM and 100 micron and 3.77 Hz for the broadband mode. The acquisition time for one line was between 4-6 minutes depending on size and orientation of the tissue sample. For the most accurate comparison between the broadband and SIM methods, both were applied in alternating lines on the same tissue section. For broadband analysis, a m/z range of 200-1500m/z was scanned, and. for SIM mode acquisition smaller m/Z windows were selected with a 15m/z range. Both modes were run with an automatic gain control (AGC) target of $5xE^5$, this value was selected to insure the same AGC target for both SIM and broadband modes without the SIM method maxing out the injection time. For SIM mode acquisition smaller m/Z windows were selected with a 15m/z range. Targeted MS2 data was also collected from the kidney tissue after each MSI run to accurately identify the analytes on the tissue.

3.4 Data Processing

Seven eicosanoids and one docosanoid were observed in the MSI experiments and identified based on the accurate mass measurement and MS/MS. Raw data were collected using Xcalibur software (Thermo Scientific) and subsequently processed using Peak-by-Peak (SpectroSwiss, Lausanne, Switzerland). Region of interest analysis was done by manually selecting them in Peak-by-Peak and averaging the spectra selected. A setting added into the Peak-by-Peak program for SIM data allowed us to image the masses in both the broadband and SIM modes from the same tissue sections for direct comparison. For data collected in alternating lines on the same tissue we used our own imaging workflow that matches each data pixel with the correct time of analysis, and ion images were generated using a custom Python script (https://github.com/hanghu1024/RAW-MSI-generator). This script uses pymsfilereader package to access mass spectra data in .RAW files. Spectral data were split by scan filters and aligned

according to scan times. A mass window of 10 ppm was used to extract intensity values for selected m/z values, which were used to generate ion images. In addition, peak intensities were normalized by corresponding total ion current (TIC) (or peak intensities of internal standard) to compensate for the signal variation during the MSI experiment. Both imaging methods use a 0-99.99% intensity scale, removing the highest 0.01% intensity pixels. The images seen in Figure 2 are normalized to the total ion current (TIC), the sizing scale bar represents 2mm on the tissue samples.

CHAPTER 4. RESULTS AND DISCUSSION

The subnanomolar concentrations of eicosanoids in mouse kidney tissue makes this lipid class difficult to study, as they get overwhelmed by the large concentrations of other lipids. By using the selected ion monitoring (SIM) mode on a Q-Exactive HF-X Orbitrap Mass Spectrometer coupled with the nano-DESI MSI source with SIM windows of m/z of 15 we have successfully increased the s/n of the identified eicosanoids, as seen in Figure 2. The eicosanoids (Table 1) identified with MS/MS spectrum matching to Metlin, found in appendix A. These include: 15-deoxy-delta-12,14-PGJ2 (m/z 315.196), 15d-PGA1 (m/z 317.212), HETE mixture of 5-, 11(R)-, and 11(s)- (m/z 319.227), HEDE mixture of 11R-, 11S-, and 15R- (m/z 323.258), 13, 14-dihydro-15-keto PGA2 (m/z 333.206), 8-iso-15-keto-PGE2 (m/z 349.201), 5-transPGF2α (m/z 353.233), a low concentration docosanoid was also identified in this group: 17R-HDoHE (m/z 343.227).

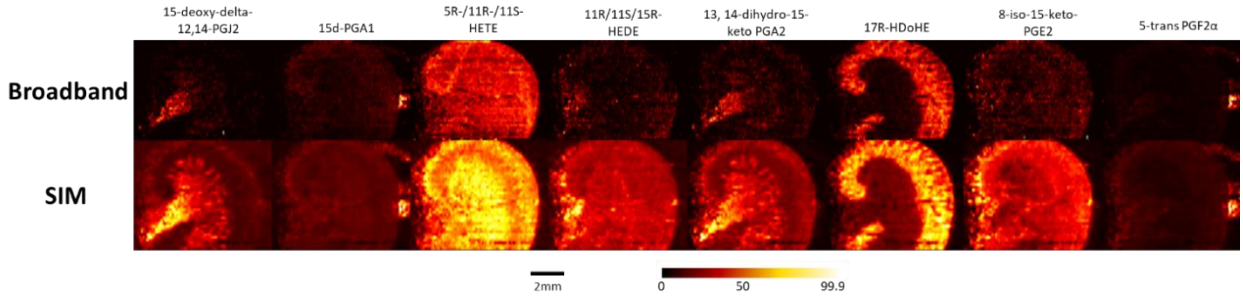

Figure 2: Comparison of broadband and SIM acquired images of MS/MS identified low concentration analytes. From left to right: 15-deoxy-delta-12,14-PGJ2 (m/z 315.196), 15d-PGA1 (m/z 317.212), HETE mixture of 5-, 11(R)-, and 11(S)- (m/z 319.227), HEDE mixture of 11R-, 11S-, and 15R- (m/z 323.258), 13, 14-dihydro-15-keto PGA2 (m/z 333.206), 17R-HDoHE (m/z 343.227), 8-iso-15-keto-PGE2 (m/z 349.201), 5-transPGF2α (m/z 353.233). Scale bar represents 2mm, color bar represents 0-99.9% intensity scale. Images normalized to total ion current (TIC) and self-normalized to their own intensity scale.

Increased levels of PGD2 and 15d-PGJ2 have been identified in the later phase of inflammation and have been associated with the resolution of inflammation.[19] Prostaglandins as a whole have been tied to many physiological and pathophysiological responses, and 17R-HDoHE has also been linked to inflammation response.[3,20] In figure 2 we see high inner medulla and renal artery localizations of 15-deoxy-delta-12,14-PGJ2 and 13,14,-dihyrdro-15-keto PGA2, suggesting links to the blood stream. There is a high cortex localization of 17R-HDoHE, the SIM images giving much more detail to this area of the kidney. 15d-PGA1, 11R/11S/15R-HEDE, 8-iso-15-

keto PGE2, and 5-trans PGF2α are seen throughout the entire tissue section, in broadband mode these likely would not be identified due to such low intensities, but they can be clearly seen in the SIM mode. We can also determine from the SIM images that 5R/11R/11S-HETE, 15-deoxy-delta-12,14-PGJ2 and 13,14,-dihyrdro-15-keto PGA2 all have suppression in the corticomedullary junction between the medulla and the cortex.

Table 1: List of low concentration analytes found on the mouse kidney tissue that have been identified through MS/MS analysis with negative mode fragment ions.

Name	**$[M-H]^-$**	**Molecular formula**	**Fragment ions**
15-deoxy-delta-12,14-PGJ2	315.196	$C_{20}H_{28}O_3$	297.18, 271.20, 59.01
15d-PGA1	317.212	$C_{20}H_{28}O_3$	273.21, 223.13, 59.01
5-, 11(R)-, 11(S)- HETE mixture	319.227	$C_{20}H_{32}O_3$	301.21, 257.22, 167.10, 113.02, 59.01
11(R)-, 11(S)-, 15R-HEDE mixture	323.258	$C_{20}H_{36}O_3$	305.24, 223.17, 199.13
13, 14-dihydro-15-keto PGA2	333.206	$C_{20}H_{30}O_4$	315.19, 271.20, 235.13, 207.10, 113.09
17R-HDoHE	343.227	$C_{22}H_{32}O_3$	325.21, 281.22, 121.10, 59.01
8-iso-15-keto-PGE2	349.201	$C_{20}H_{30}O_5$	331.19, 287.20, 229.01, 59.01
5-trans-PGF2α	353.233	$C_{20}H_{34}O_5$	335.22, 317.21, 309.20, 235.13, 171.10, 59.01

From initial findings, we have obtained an average of 100 fold increase in signal to noise between the broadband and SIM analysis of the selected analytes using the lowered AGC target. In previous runs with an AGC target of $3E^6$ the SIM scans were not reaching the target in the given injection time, thus not reaching the same number of ions of the selected masses. These results, while still showing significant differences between the SIM and broadband, were not as directly comparable and gave an average of 4 fold increase in s/n. This project is still a work in progress, as follow-up results are still being obtained and analyzed.

CHAPTER 5. CONCLUSION

Using selected ion monitoring has proven to enhance the ratio of signal to noise in a way to clearly identify the localizations of low concentration lipid classes on tissue. With an increase in signal to noise level in the SIM mode of 100x compared to the broadband mode, the images created show a significant difference in clarity and more detailed information on localizations of the analytes in the mouse kidney tissue.

Future work will focus on looking into what level of signal increase can be seen with a targeted MS/MS approach.[21] While a higher s/n than the SIM mode is expected, the time frame for a full MS/MS imaging experiment will greatly exceed that of the more targeted SIM mode imaging run. The goal will be to determine whether the increase in s/n is enough to make the longer run time worth it. The fragmentation of MS/MS may also decrease the signal seen of the parent ion peaks, so this will also need to be taken into consideration.

Another direction for the eicosanoids in particular is to continue to decrease the size of the SIM windows, the s/n will continue to increase, ideally having a window of 1 amu would produce the highest s/n ratio. Our group is also working on increasing the signal of eicosanoids by changing the solvent composition by adding ammonium formate and ammonium fluoride.

APPENDIX A

MS/MS spectra of identified analytes

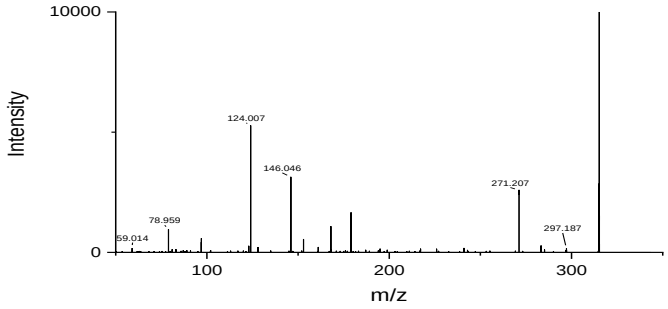

Figure 1A: m/z 315.197

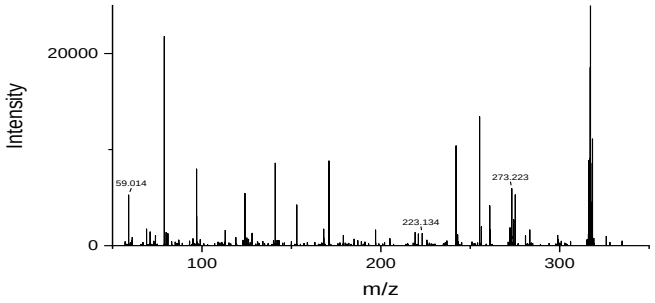

Figure 2A: m/z 317.212

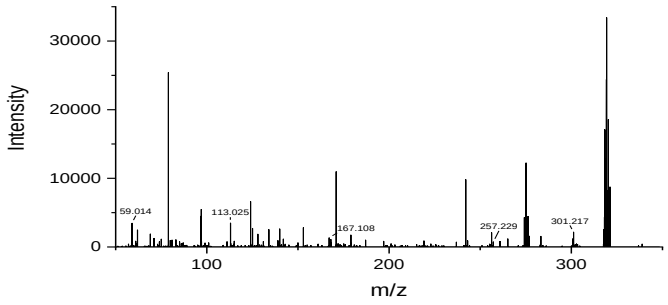

Figure 3A: m/z 319.228

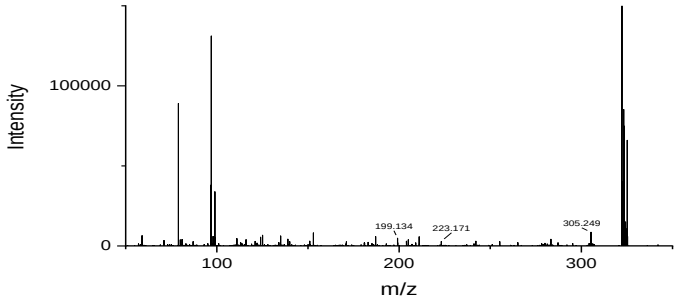

Figure 4A: m/z 323.259

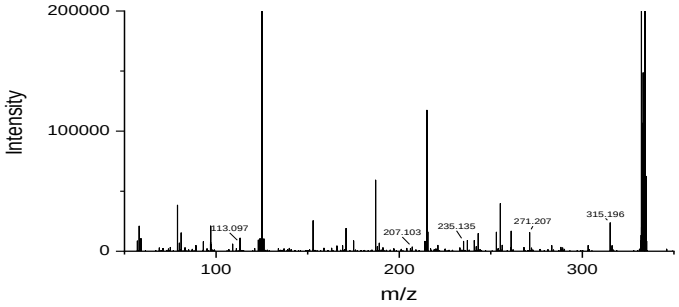

Figure 5A: m/z 333.207

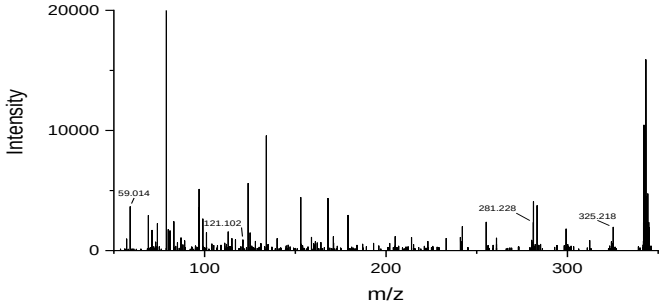

Figure 6A: m/z 343.228

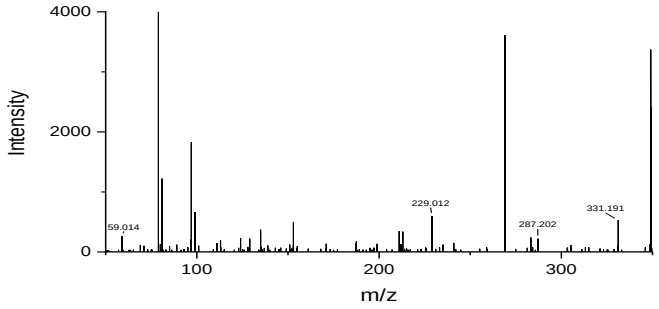

Figure 7A: m/z 349.202

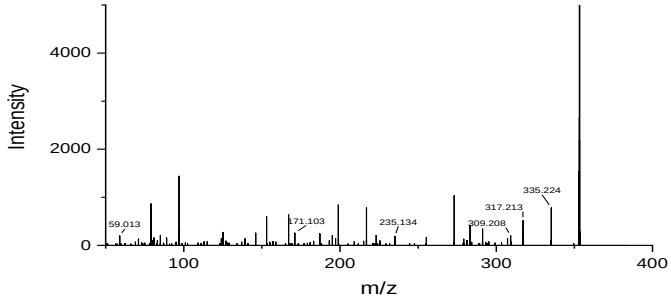

Figure 8A: m/z 353.233

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Bellarmine University
- Advisor: Dr. Joseph Sinski — August 2013-May 2015
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  - "Discovery and Analysis of Alien Organic Materials in and Around the Ohio River in Louisville, KY"
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- Perfected skills and techniques of separation of solid materials with liquid extractions.
- Received Clariant Corporation Endowed Award in 2014

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Toxicology Quality Lead — October 2017-June 2018
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- Maintain required record keeping in compliance of CLIA/COLA laboratory regulations.
- Assist COLA inspector with laboratory surveys.
- Assist with writing and improving SOPs.
- Improve processes to support the growing of the laboratory.
- Manage corrective actions.

- In charge of obtaining and maintaining employee documentation of qualifications and competency.
- In charge of assuring proficiency for all testing performed on patient specimens in the laboratory.
- In charge of securing the privacy of PHI and ePHI according to HIPAA guidelines and regulations.

Toxicology Analytical Chemist October 2015-June 2018

- Operation, and maintenance of Waters Xevo TQS/TQD MS/MS, Acquity I-Class UPLC/BSM and
  other laboratory instrumentation as needed (AU680, liquid handlers, etc)
- Achieving and maintaining all COLA regulations for certification and accreditation.
- Maintains clean laboratory work environment.
- Prepares calibrators, internal standards, QCs and solvents.
- Documents solution preparation and instrument maintenance
- Insuring proper calibration/working order of laboratory equipment and computers.
- Performs extraction of patient samples for the 63-analyte confirmation panel, oral fluid confirmation panel, and
  meth D/L confirmation panel in accordance with SOPs.
- Performs all steps of sample review
- Monitors environmental data including temperatures, humidities, etc
- Work under pressure and meet deadlines, while maintaining a positive attitude
- Ability to work independently and to carry out assignments to completion within parameters of instructions given, prescribed routines, and standard accepted practices.
- Ability to problem-solve and troubleshoot under pressure.
- Knowledge of the principles and techniques utilized in chemistry, biology and biochemistry, physiology or physics applicable to toxicology laboratory procedures

Bellarmine University Department of Chemistry
Lab Assistant May 2013 - August 2014

- Further understanding of lab techniques
- Experience working with hazardous chemicals
- Maintaining clean lab areas

Academic Achievements

KEES Scholarship (2011 - 2015); Monsignor Horrigan Scholar (2011 - 2015); Trustee Scholarship (2011 - 2015);
Clariant Corporation Endowed Award (2014 - 2015);
Bellarmine Chemistry Club Vice President (2014 - 2015);
American Chemical Society, Louisville Section, Award for Outstanding Chemistry Graduate from Bellarmine University, (2015)

Further Laboratory Experience

Bellarmine University

- Up to four years of continued use and gained knowledge of instruments including:
  - Proton, C^{13}, DEPT, COSY, and HETCOR NMR (ANASAZI FTNMR 90MHz)
  - Infra-Red Spectrometer (JASCO FT/IR 490 plus and 4200)

    - Gas Chromatography Mass Spectrometer-QP5000 (Shimadzu GC17A and GCMS-QP5000)
    - Atomic Absorption Spectrometer
    - High Performance Liquid Chromatography
    - UV-vis Spectromter
    - JASCO P1010 Polarimeter and Flourimeter
- High level of experience on computer software (i.e. Microsoft Word, Excel, Mathcad, Spartan, Powerpoint, etc.)