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# High-Resolution Ion Mobility Analysis of Isomeric Bile Acids Using Structures for Lossless Ion Manipulations (SLIM) IM-MS

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# HIGH-RESOLUTION ION MOBILITY ANALYSIS OF ISOMERIC BILE ACIDS USING STRUCTURES FOR LOSSLESS ION MANIPULATIONS (SLIM) IM-MS

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**A Thesis**
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**Clemson University**

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**In Partial Fulfillment**
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**Masters in Chemistry**

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**by**
**Emmaleigh Efird**
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**Accepted by:**
**Dr. Christopher D Chouinard, Committee Chair**
**Dr. Kenneth Marcus**
**Dr. Daniel Whitehead**

## ABSTRACT

Bile acids are an essential part of the human digestion system and play an important role in lipid absorption, bacteria movement in the small intestine, and regulation of the farnesoid X receptor (FXR) and G protein-coupled bile acid receptors (GPBAR) responsible for homeostasis. They are synthesized in the liver, stored in the gallbladder and the excess is carried on to the small intestine. Many liver and intestinal diseases are diagnosed based on bile acid metabolism pathways and the structures of those bile acids. Therefore, the bile acid structure determines the function of the molecule. Many bile acids are similar in structure and only differ by an orientation of a hydroxyl or other group. This difference can be the key to diagnosing a patient with a lifechanging disease.

In analytical chemistry, ion mobility mass spectrometry (IM-MS) is used to measure the difference in size and shape of the molecules by calculating the collision cross-section (CCS). In this study, we aim to analyze different bile acids using Structures for Lossless Ion Manipulations (SLIM) ion mobility and comparing nitrogen and helium buffer gas to determine the best method of separation. The calculated CCS values were analyzed to determine trends that would accurately identify these bile acids.

Additionally, this method will be applied to test a clinical assay for its sensitivity. A concentration study will be performed on specific bile acids that are used in the total bile acid test to be applied in clinical settings. This test is important to improve patients' diagnosis and have quicker results than the typical laboratory test. The goal is to increase scientific knowledge of ion mobility in the clinical field, not only to further biological analysis, but also to enhance clinical care around the world.



## KEYWORDS

Bile acids, ion mobility, mass spectrometry, collision cross section, isomers

## DEDICATION

This thesis is dedicated to my friends and family. I am forever grateful to my parents who guided me and encouraged me during my time in graduate school. They were available to listen to me when I wanted to give up and checked in on me even when I did not want to talk. I am also thankful for their prayers they have offered me all the years and countless blessing they have given to me over the years. To my friends, who are always by my side, it means the world to me that our friendship is still strong after all these years. I appreciate everyone who I have crossed paths along the way and I could not be here without you.

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I am grateful to so many people for their support and patience during this time. First, I would not be here without God as I give all glory to him for giving me the opportunity to be able to do this.

I am extremely grateful to Dr. Chouinard's lab for allowing me to perform research in the group. To Dr. Chouinard, you have opened my mind to the world of mass spectrometry and been my mentor and teacher the past two years. I have developed a fascinating interest in this field and owe it all to you. I am thankful for your mentorship as I navigate this process and I hope to carry what I have learned into my future career. To my lab mates, Ralph, Shadrack, Heidi, Terra and the undergraduate students, I have loved getting to know each of you and wish you the best wherever you go. Finally, I could not have done this work without the financial support from Agilent Technologies, MOBILion Systems, Inc., and Clemson University startup funds.

To my professors, it has been an honor to work with you and learn all about your field in chemistry. Special thanks to Dr. Marcus and Dr. Whitehead for being on my committee.

It has been a pleasure to be a part of Clemson Chemistry and I will cherish all the memories as I plan for the next journey.

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## CHAPTER 1: INTRODUCTION

According to an article by Rui Wang et. al., digestive diseases affect more than 40 million people in the United States and account for millions of doctors' visits and health care bills.[1] As a result, there is a strong desire to correctly diagnose gastrointestinal and liver diseases by understanding the safe and harmful concentrations of isomeric bile acids. With this research, we developed a rapid method using Structures for Lossless Ion Manipulations Ion Mobility Mass Spectrometry (SLIM-IM-MS) for a targeted quantification of bile acids. SLIM is the optimal method of separation compared to liquid chromatography–mass spectrometry (LC–MS) since there is higher resolution of isomers and faster separation times; within a few seconds.[2,3] This method determined the selectivity of certain bile acids and were analyzed to indicate any form of disease. The first project analyzed thirty-one bile acids in the Drift Tube-Ion Mobility-Mass Spectrometry (DT-IM-MS) and SLIM instruments and determine their collision cross section (CCS) which helped identify them. We chose bile acids such as chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) which are two that differ in the position of a single hydroxyl group. The next project was able to determine the concentrations of bile acids that indicate specific diseases. Since bile acids are involved in the metabolic pathway, they interact with other molecules and certain enzymes, such as inflammatory markers and cholesterol 7α-hydroxylase (CYP7A1). Therefore, concentration levels are important for metabolic function. There are four specific bile acids that were targeted that are primarily linked to liver disease. We hope to develop a method that will be practical in a clinical application for quantification of bile acid mixtures in representative biological samples (i.e., urine).

Many people have suffered from digestion issues, and they need solutions to mitigate the problems or alleviate their symptoms entirely. By performing a quantitative and qualitative analysis of bile acids, we studied the connection between liver disease and the concentration of bile acids. We effectively demonstrated the harmful concentrations of bile acids in patients with liver disease. In the future, we hope to deliver more rapid and accurate results than the typical bile acid tests in clinical settings so that doctors can more effectively diagnose their patients. Understanding the function of bile acids will help enhance the study of metabolic pathways, lead to new drug developments, and increase diagnostic biomarkers for more effective patient care.

**Biological Importance**

Bile acids are essential to the human body, known for their role primarily in the digestive system. However, bile acids contribute to many physiological functions because of their versatility from being an amphipathic, steroidal structure. Besides the digestive system, bile acids provide support in the nervous and immune systems. For the digestive system, they are responsible for the absorption of fats and the regulation of the liver, as well as the metabolism of microorganisms in the intestines. Bile acids also act as signaling molecules to bile acid receptors such as the FXR, G protein-coupled bile acid receptor 1 (GPBAR1), and vitamin D receptor (VDR), which greatly influence the inflammatory responses of the immune system.[4] These receptors are connected to the central nervous system which interact with bile acids to create neurotransmitter activity such as anxiety responses.[4] Bile acids are a cause for many diseases because of their involvement in metabolism and other biological systems.

A significant impact of bile acids is the gut microbiota which is responsible for many health issues. Bile acids pool size has been linked to gut metabolism and studies are being performed to monitor their interactions. They have a mutual connection, so bile acids and the gut microbiota work together to establish a healthy environment.[5] A major disease is cirrhosis, liver disease, which is linked to low bile acid levels from the small intestine due to an increase in bacterial dysbiosis.[6] Additionally, bile acids are strongly associated with dietary fiber which bind together to metabolize into secondary bile acids. Many metabolism disorders can be attributed to a lack of fiber and an imbalance in the gut bacteria.[7] Although not much is known about the specific molecules that cause these digestive diseases, there are plenty of studies to support the importance of a high fiber and low-fat diet.

One major issue with bile acids is they all have a similar structure with a few differences in their functional groups. The structures in Figures 1-1 to 1-6 illustrate the minor differences in the position of the hydroxyl groups of the isomers tested in the experiment. Differentiating between bile acid isomers will allow for more accurate diagnosis of diseases and a better understanding of their causes for preventative treatments.

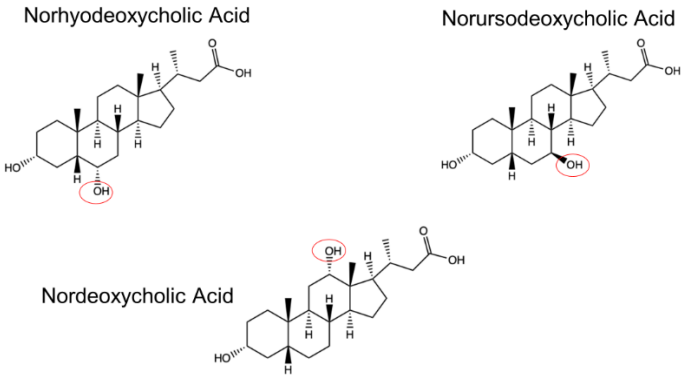



***Figure 1-1:*** *Isomeric group of norhyodeoxycholic acid, norursodeoxycholic acid, and nordeoxycholic acid showing the primary chemical functionality which differ in their hydroxyl group.*

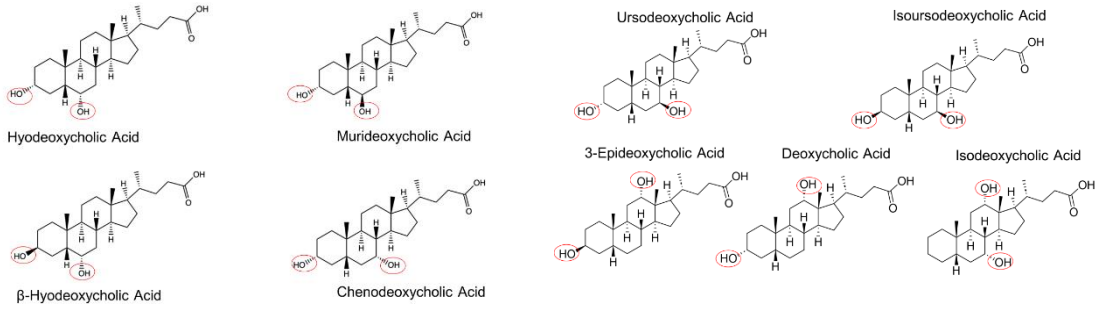



***Figure 1-2:*** *Isomeric group of hyodeoxycholic acid, murideoxycholic acid, β-hyodeoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, isoursodeoxycholic acid, 3-epideoxycholic acid, deoxycholic acid, and isodeoxycholic acid showing the primary chemical functionality which differ in their hydroxyl groups.*

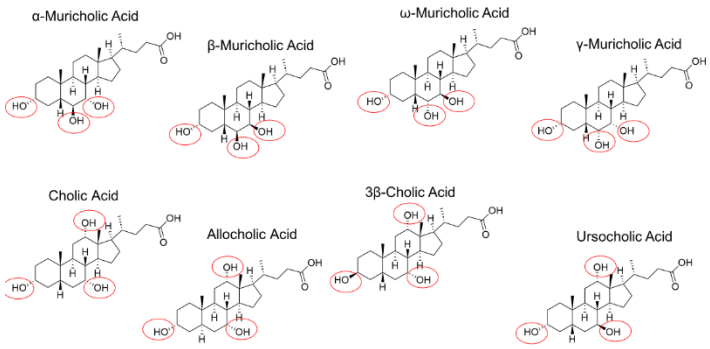



***Figure 1-3:*** *Isomeric group of α-muricholic acid, β-muricholic acid, ω-muricholic acid, γ-muricholic acid, cholic acid, allocholic acid, 3β-cholic acid, and ursocholic acid showing the primary chemical functionality which differ in their hydroxyl groups.*

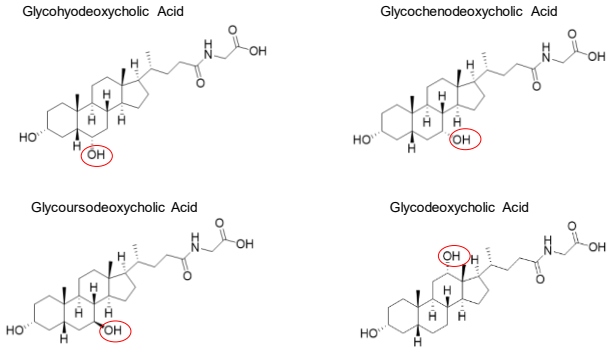



***Figure 1-4:*** *Isomeric group of glycohyodeoxycholic acid, glycochenodeoxycholic acid, glycoursodeoxycholic acid, and glycodeoxycholic acid showing the primary chemical functionality which differ in their hydroxyl group.*

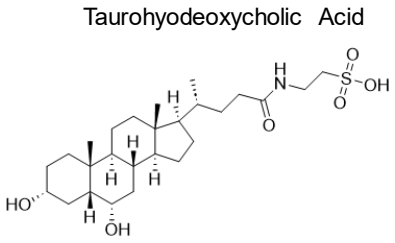



***Figure 1-5:*** *Taurohyodeoxycholic acid structure with no isomers.*

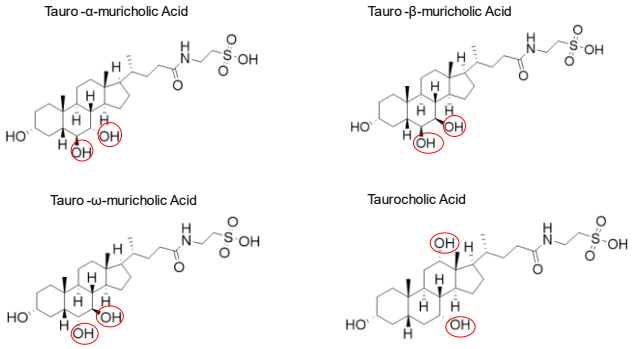



***Figure 1-6:*** *Isomeric group of tauro-α-muricholic acid, tauro-β-muricholic acid, tauro-ω-muricholic acid, and taurocholic acid showing the primary chemical functionality which differ in their hydroxyl groups.*

Because of the vast number of structures, it is necessary to understand the metabolic pathway of bile acids and their functions. Bile acids are synthesized in the liver from cholesterol. Then they travel to the intestines where they are reabsorbed in the ileum and returned to the liver.[8] They are divided into primary and secondary categories based on their place of digestion. Primary bile acids are synthesized in the liver and secondary ones are produced by intestinal bacteria. Examples of primary bile

acids are cholic acid (CA) and CDCA. These primary bile acids are conjugated or deconjugated with amino acids, glycine or taurine, to help with solubility. Once these conjugated bile acids are released into the small intestines, they are converted to the secondary bile acids such as DCA. This process is known as enterohepatic circulation. During this process, bile acids are regulated by hepatic uptake to ensure the body systems are functioning properly.

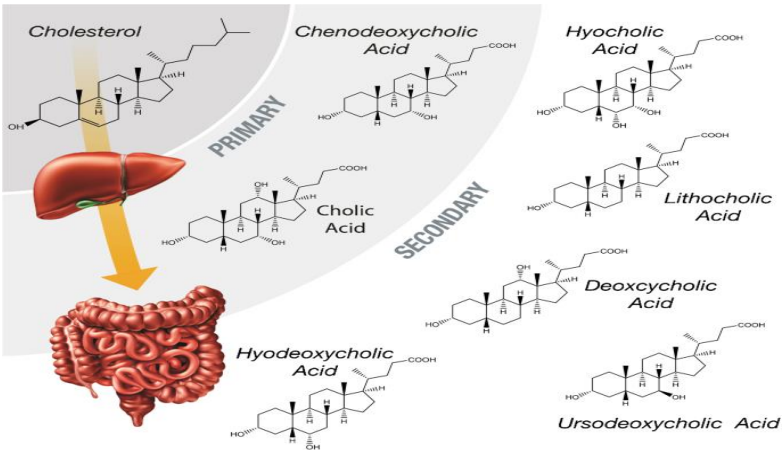



***Figure 1-7:*** *A schematic of the metabolic pathway that transforms cholesterol into primary and secondary bile acids through the liver and small intestine.*[9]

## Current Methods

Different analytical techniques that are used to identify bile acids are liquid chromatography - mass spectrometry (LC-MS) and immunoassays. While these techniques have been effective at detecting biological compounds, they lack the capability to detect isomeric and isobaric species in a timely manner.

One common method in identifying bile acids is LC-MS which is a versatile separation technique targeted for an analyte. Typically, LC-MS has been effective at separating bile acids and providing structural information. Most LC-MS methods rely on

reverse phase-chromatography and atmospheric pressure ionization.[10] However, problems occurred with sensitivity and speed. With the creation of electrospray ionization (ESI), there have been improvements made for better analysis.[11] These methods are great for biological samples, but they are time consuming and not suited for conjugated bile acids.[12]

The next method is immunoassays which work by using an antibody to target an analyte. Many of these tests are used in clinical settings because of their rapid and simple approach. A type of immunogen is the bile acid-bovine serum albumin (BSA), which is commonly used in radioimmunoassays and conditions can be altered for quantification of bile acids.[13] The cross-reactivities of each bile acid compound are different based on their linkages to the carrier protein. This technique works well for single bile acids and would not be effective for a comprehensive analysis. Bile acids are very specific in their structure and require a technique more selective than the immunoassays.

**Challenges for Bile Acid Analysis**

These methods have proven effective in many scenarios, however, there are challenges that arise in detecting bile acids. Some of these challenges include detecting isomers and the time frame of separation. One problem with bile acid detection is that there are several isomers that differ in the stereochemistry of hydroxyl groups, which can lead to inadequate structure identification.[14] Even with options like MS/MS and high- or low-energy CID isomer differentiation, biological mixtures can be difficult as it is probable that the $[M-H]^-$ ion itself is a composite peak comprising not only chemical isomers of the target molecule but also of other compounds with the same nominal

mass.[10] With bile acids, LC-MS may have to be used in addition to derivatization such as by Girard's reagents and 1,1-carbonyldiimidazole (CDI)[15,16] to get confirmation of the compounds.[16] These metabolites can be the difference between a normal, healthy patient and a colon cancer patient. Another issue is that LC-MS is time consuming for effective separation. These LC-MS methods are effective yet require 10-35 mins for isomers.[9] An alternative method that would enhance bile acid separation is ion mobility because it can detect structural differences in a millisecond time scale.

**Ion Mobility and Bile Acids**

A novel technique called ion mobility can detect isomers based on their size, shape, and charge to determine a collision cross section (CCS) value that is unique to each bile acid. This value is determined for every molecule using the Mason-Schamp equation which describes the collision of the ion in the drift gas and can give information about the conformation of the molecule.[17] Because of this, the drift gas identity is important for the calculation of the CCS value among other variables. Ion mobility has been used in several applications for detecting molecules. Studies have targeted molecules such as glucocorticoids[18], steroid hormones,[15] organic micropollutants[19], and fentanyl isomers[20]. These have several implications in the clinical, environmental, and forensic fields for the future.

By using ion mobility, isomeric bile acids can be detected to develop a rapid and effective method for diagnosing patients. Previously there have been studies to indicate the presence of bile acids using ion mobility, specifically using DTIMS. As shown in Figure 1-8, the analyte molecules go through an ESI source and then ions pass through an inlet gas capillary, focused by a high-pressure funnel. The ions are accumulated in

the ion trap funnel and then travel in a 78 cm tube by a weak electric field. The ions exit through a rear ion funnel to be detected by the QTOF MS.

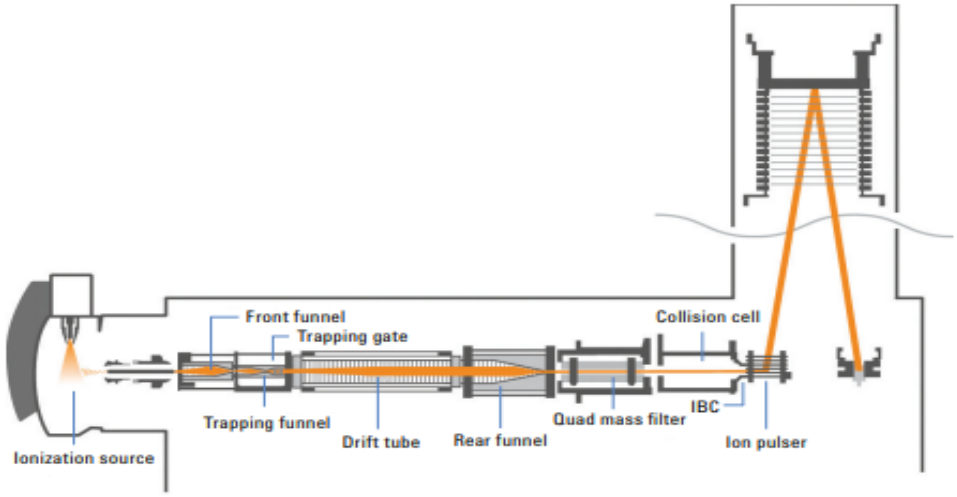



***Figure 1-8:*** *Schematic of the Agilent 6560 IM-QTOF instrument used. The instrument contains a 78 cm drift tube maintained at 4 Torr nitrogen at 25 °C.*

One study by Poland, et. al analyzed bile acids and their derivatives to identify changes in metabolism from biliary diversion surgery.[21] They used ion mobility with liquid chromatography to rapidly detect bile acids with little preparation and cleanup. Their method is crucial to analyzing gastrointestinal diseases from biological samples. Additionally, Zheng et al. explored bile acid's structure using drift tube ion mobility.[9] Developments in ion mobility have led to many molecules, such as bile acids, to be detected in a faster and more efficient manner.

### Effects of Changing Gases

A fundamental part of ion mobility is the drift gas used in the instrument. Much like the stationary phase in liquid chromatography, the drift gas composition can

enhance separations. The mass of the drift gas is a component of the CCS calculations which can be used to optimize chemical selectivity and resolution to achieve reproducible data values.[22] These CCS values correlate to each drift gas and are represented by a nomenclature to denote which gas was used.[23] Several gases can be used including argon, helium, nitrogen, and carbon dioxide. The one requirement for a gas is it must be chemically inert for the thermal collisions. One study evaluated these gases to achieve quantitatively reproducible CCS values.[22] They experimented with different conditions and determined the optimal parameters which provided the highest reproducibility of the CCS values. As a result, they discovered that nitrogen has the highest reproducibility across many compounds that require high resolving power and peak capacity. While argon has the highest peak capacity, it is not as high in the higher mass ranges compared to nitrogen.

Additionally, the drift gas is targeted to the analyte and needs to be reliable. Since, the analytes here are bile acids, the chosen drift gases were nitrogen and helium. Nitrogen was proven to work for high mass ranges, like bile acids, with the highest reproducibility.[22] A study on bile acids explored trends in different drift gases by measuring CCS values.[3] Other than nitrogen, helium was discovered to have the lowest resolving power and performed the least in isomer separation. Other gases, such as carbon dioxide and argon did have better resolution, however helium is known to be accurate to the published CCS values.[22] By focusing on the CCS values, helium is a great option for consistent values to the literature. For ion mobility experiments, drift gases are an important factor that will affect the performance of the ion mobility separations and reproducibility of CCS values.

**Advanced Techniques-SLIM**

Ion mobility is being used in many instruments including DT-IM-MS and SLIM-IM-MS. The drift tube instrument has a 1 meter long tube that ions pass through in a static electric field. In contrast, the SLIM has a 13 meter long serpentine path where the ions pass through in a travelling wave dynamic electric field. The main difference is the SLIM-IMS has a much longer path and, therefore, has increased resolution for isomers. To generate a standard CCS value, the drift tube is used first and then the SLIM is used to further enhance the separation. Ion mobility is essential in differentiating between bile acids and will be the future of clinical diagnostics by providing faster and more accurate results than the typical bile acid tests.

One of these new techniques is SLIM which was developed by Pacific Northwest National Laboratory (PNNL) and commercialized by MOBILion systems.[24] As mentioned earlier, SLIM has a longer separation path that is beneficial in separating isomers. Previous studies in the Chouinard group have used SLIM on cannabinoid[24], vitamin D[25] and xylazine metabolites.[26] In this project, SLIM was used with nitrogen and helium gas to optimize separation of isomeric bile acids for future clinical applications.

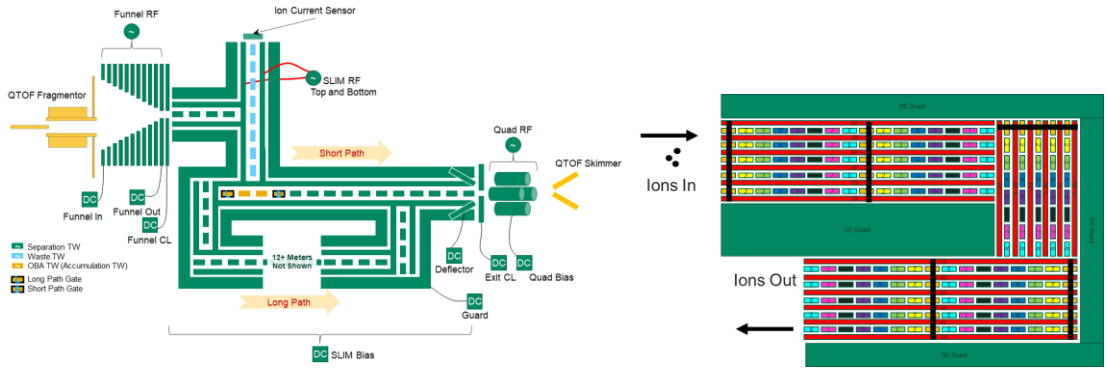



***Figure 1-9:*** *Schematic of the MOBILion TW-SLIM system. This instrument features a 13 m SLIM design maintained at 2.5 Torr buffer gas and approximately 25 °C.*

# CHAPTER 2: EXPERIMENTAL METHODS FOR BILE ACID ANALAYSIS USING ION MOBILITY MASS SPECTROMETRY

## Introduction

For the first project, thirty-one bile acids were analyzed using DT-IM-MS and SLIM-IM-MS by calculating their CCS values. These bile acids are grouped into isomers, and they were compared accordingly. For instance, there are three bile acids with a molecular weight of 378.6 g/mol. A method was developed on the DT and SLIM instruments to enhance the selectivity and sensitivity of these bile acids. This is the first application of SLIM on isomeric bile acids. The variables that were changed, specifically, are the drift gases and the mode of ionization. The ions behavior was different in each drift gas and in positive or negative mode because of their density and diffusion rates.

To continue the detection of bile acids, the second project was to develop a method to increase the sensitivity of bile acids for clinical applications. The bile acids included in this experiment are CA, CDCA, DCA, and UDCA, which are targeted in the

total bile acids test according to Associated Regional and University Pathologists, Inc. (ARUP) Laboratories. This assay is performed for liver function and intrahepatic cholestasis of pregnancy as well as patients in bile acids therapy. Bile acid levels greater than 40 μmol/L are associated with fetal complications.[27] The problem with this test is the bile acids can only be detected at limiting concentrations like CA is detected at 1.9 μmol/L. The goal is to use SLIM-LC-MS to be able to detect these bile acids at lower concentrations. To measure the sensitivity, a series of bile acids was injected in the SLIM at varying concentrations to see if this instrument has improved detection than the assay.

**Chemicals and Reagents**

Bile acids were obtained from Cayman Chemical (Ann Arbor, MI) and included norhyodeoxycholic acid (NHDCA), norursodeoxycholic acid (NUDCA), nordeoxycholic acid (NDCA), hyodeoxycholic acid (HDCA), murideoxycholic acid (MDCA), β-hyodeoxycholic acid (β-HDCA), chenodeoxycholic acid (CDCA), ursodeoxycholic acid (UDCA), isoursodeoxycholic acid (IUDCA), 3-epideoxycholic acid (3-EDCA), deoxycholic acid (DCA), isodeoxycholic acid (IDCA), α-muricholic acid (α-MCA), β-muricholic acid (β-MCA), ω-muricholic acid (ω-MCA), γ-muricholic acid (γ-MCA), cholic acid (CA), allocholic acid (ACA), 3β-cholic acid (3β-CA), ursocholic acid (UCA), glycohyodeoxycholic acid (GHDCA), glycochenodeoxycholic acid (GCDCA), glycoursodeoxycholic acid (GUDCA), glycodeoxycholic acid (GDCA), glycohyocholic acid (GHCA), glycine-β-muricholic acid (G-β-MCA), taurohyodeoxycholic acid (THDCA), tauro-α-muricholic acid (T-α-MCA), tauro-β-muricholic acid (T-β-MCA), tauro-ω-

muricholic acid (T-ω-MCA), taurocholic acid (TCA). Solutions of methanol and water (0.1% formic acid) were Fisher Scientific Optima LC-MS grade (Pittsburgh, PA).

**Sample Preparation**

The 31 bile acid stock solutions were prepared from a powder in 50% (v/v) aqueous methanol at a concentration of 1 mg/mL. Each solution was stored in the freezer at -20 °C. Then each stock solution was diluted in 50% (v/v) aqueous methanol to make solutions of 5 µg/mL. For the concentration experiment, 4 bile acids (CA, CDCA, DCA, UDCA) were prepared in concentrations ranging from 0.08 µg/L to 20.4 µg/L in increasing intervals. The solutions were prepared from the stock solutions and diluted in 50% (v/v) aqueous methanol.

**Instrumentation**

For the initial experiment, all analyses were first performed in triplicate using an Agilent 6560 drift tube (DT) IM-QTOF (Santa Clara, CA) for direct measurement of collision cross section (CCS). Samples were introduced via direct injection (10 µL) with a flow rate of 0.300 mL/min of (50:50 (v/v) water (0.1% formic acid)/methanol) using an Agilent 1290 Infinity II UHPLC. Ionization was performed with an Agilent Jetstream (AJS) ESI source in positive and negative mode, and the MS data were acquired using full scan mode. The drift tube was maintained at approx. 3.95 Torr nitrogen and approx. 27 °C, and the electric field strength was 18.5 V/cm. Additional runs were performed in helium in positive and negative mode. Further instrument parameters can be found in Table 2-1. After performing the initial runs in nitrogen and helium, the varying concentrations of the 4 bile acids were directly injected (10 µL) into an Agilent ZORBAX

Extend-C18 column (2.1 × 50 mm, 1.8 µm) on the DT at 28.3 °C. The mobile phase consisted of water containing 0.1% formic acid and methanol. The flow rate was set at 0.300 mL/min with a total runtime of 10 minutes.

Analyses were also performed in triplicate using a MOBIE HRIM SLIM (MOBILion Systems, Inc., Chadd's Ford, PA) coupled to an Agilent 6546 QTOF (Santa Clara, CA). Samples were introduced via direct injection (10 µL) with a flow rate of 0.300 mL/min of (50:50 (v/v) water (0.1% formic acid)/methanol) using an Agilent 1290 Infinity II UHPLC. Ionization was performed with an Agilent Jetstream (AJS) ESI source in positive and negative modes as well as nitrogen and helium gas. The SLIM device was maintained at 2.50 Torr nitrogen gas and 25 °C. SLIM traveling wave (TW) parameters were first optimized in short path (0.4 m) IM mode, and then separations and analysis were performed using HRIM (13 m) mode. Briefly, these conditions included a 100 ms trapping time and SLIM TW operated at 20 kHz frequency and 40 $V_{pp}$ amplitude; based on the electrode geometry, this frequency equates to a TW speed of 315 m/s. Further details of the SLIM parameters can be found in Table 2-2.

*Table 2-1:* ***Instrumental parameters for the Agilent 6560 IM-QTOF.***

| Instrument Region | Instrumental Parameter | Experimental Value |
|---|---|---|
| **Source** | Gas Temp | 325 °C |
| | Drying Gas | 12 L/min |
| | Nebulizer | 20 psi |
| | Sheath Gas Temp | 275 °C |
| | Sheath Gas Flow | 10 L/min |
| | VCap | 4000 V |
| | Nozzle Voltage (Expt) | 1000 V |
| | Fragmentor | 400 V |
| | Oct 1 RF Vpp | 750 V |
| **High Pressure Funnel** | High Pressure Funnel Delta | 150 V |
| | High Pressure Funnel RF | 200 V |
| **Trapping Funnel** | Trap Funnel Delta | 180 V |
| | Trap Funnel RF | 200 V |
| | Trap Funnel Exit | 10 V |
| | Entrance Grid Delta | 10 V |
| | Entrance Grid Low | 96 V |
| | Entrance Grid High | 107 V |
| | Trap Entrance | 91 V |
| | Trap Exit | 90 V |

| | | |
|---|---|---|
| | Trap Fill Time | 3900 µs |
| | Trap Release Time | 150 µs |
| | Trap Exit Grid 1 Delta | 4 V |
| | Trap Exit Grid 1 Low | 86.3 V |
| | Trap Exit Grid 1 High | 90.3 V |
| | Trap Exit Grid 2 Delta | 8.5 V |
| | Trap Exit Grid 2 Low | 85.8 V |
| | Trap Exit Grid 2 High | 94.3 V |
| **Drift Tube** | Drift Tube Entrance Voltage | 1700 V |
| | Drift Tube Exit Voltage | 250 V |
| | Drift Tube Field Strength | 18.6 V/cm |

***Table 2-2**: Instrumental parameters for the MOBILion MOBIE SLIM & Agilent 6546 QTOF.*

| Instrument Region | Instrumental Parameter | Experimental Value |
|---|---|---|
| **Ionization Source** | Polarity | Positive |
| | Gas Temp | 325 °C |
| | Drying Gas | 12 L/min |
| | Nebulizer | 20 psi |
| | Sheath Gas Temp | 275 °C |
| | Sheath Gas Flow | 10 L/min |
| | VCap | 4000 V |
| | Nozzle Voltage (Expt) | 1000 V |
| | Fragmentor | 400 V |
| | Oct 1 RF Vpp | 750 V |
| **SLIM Conditions** | Funnel In | 165 V |
| | Funnel Out | 100 V |
| | Funnel Conductance Limit (CL) | 95 V |
| | SLIM Bias | 90 V |
| | SLIM Mode | HRIM (13 m) |
| | SLIM Wave Shape | Sine |
| | Fill Time | 100 ms |
| | Trap Time | 0.3 ms |

| | | |
|---|---|---|
| | Release Time | 3.2 ms |
| | IMS Frame Length | 800 ms |
| | Fill TW Frequency | 15 kHz |
| | Fill TW Amplitude | 5 $V_{pp}$ |
| | Release TW Frequency | 15 kHz |
| | Release TW Amplitude | 30 $V_{pp}$ |
| | Separation TW Frequency | 20 kHz |
| | Separation TW Speed | 180 m/s |
| | Separation TW Amplitude | 40 $V_{pp}$ |
| | SLIM Exit CL | 50 V |
| | Quad Bias | 45 V |
| | Quad Pressure (Rough Vac) | 2.5 Torr |
| | SLIM RF Amplitude | 270 V |
| | SLIM RF Frequency | 1200 kHz |

**Data Processing and Analysis**

Acquisition of the IM-QTOF data was performed using Agilent MassHunter B.09.00 (Build 9.0.9044.0) and visualized/processed in Agilent IM-MS Browser 10.0.1 (Build 10.0.1.10039). DT CCS values were measured using the established single-field method[7] based on beta and t-fix values for the Agilent Tune Mix ions. SLIM data acquisition was performed using Agilent MassHunter Acquisition Version 11.0 (Build 11.0.221.1) and MOBILion EyeOn software (0.0.2.2619-release-1.5.14.3). All SLIM data

was first converted from the MOBILion .mbi file format into Agilent .d file format using the EyeOn software, and then pre-processed using the PNNL PreProcessor 4.0 (2022.02.18) (Richland, WA) with a drift bin compression of 3:1 and CCS conversion.10 Further data visualization/processing was then performed using Agilent IM-MS Browser 10.0.1 (Build 10.0.1.10039). CCS measurements were made by first calibrating the SLIM system using the Agilent Tune Mix ions, which allowed for conversion of arrival time to CCS using the PNNL PreProcessor.[29,30] Briefly, this calibration process involved plotting of experimental arrival times for the Agilent Tune Mix ions (acquired using experimental parameters identical to those used for measurement of the bile acid compounds) against their corresponding reduced $^{DT}CCS_{N2}$ values, which allowed for creation of a third order polynomial calibration curve.[31] Experimental arrival times for the bile acids could then be converted into reduced CCS and then (using charge and reduced mass) into conventional $^{SLIM}CCS_{N2}$. For the helium values, this system was repeated using tune mix values for the helium gas. Further details of this calibration process are found in a recent paper by Rose et al.[29]

The concentration was extracted from the chromatographs to find the peak area for each analyte. The peak area was determined for a set range of drift times and m/z for each analyte to stay consistent. Then the peak area vs. concentration was graphed on a calibration curve to determine a line of best fit and $R^2$ value. The method was successful because the concentration detected was less than the amount indicated in the ARUP total bile acids assay.

## Results and Discussion

The data collected was analyzed in the DT-IM-MS and SLIM-IM-MS in positive or negative mode with either helium or nitrogen gas. The results demonstrated varying degrees of separation for the different isomer groups. The positive mode with nitrogen yielded the best separation overall because the peaks were further apart which means better resolution. Given the CCS values, each bile acid could be evaluated to determine the best drift gas and positive or negative mode for each one. Additionally, the concentration experiment was effective as the concentrations were detected and fit to a calibration curve. Based on these results, the bile acids were effectively analyzed to be tested in a clinical setting.

### Analysis of Bile Acid Separation with Different Parameters

For this project, different variables were testing to determine an effective method to separate isomeric bile acids. First, the DT and SLIM were testing for all the bile acids. As seen in Figure 2-1, the bile acids had decent separation in the drift tube and even better separation in the SLIM. This is due to the longer path of separation where the smaller ions can escape quickly. This pattern was noticeable throughout the experiment as SLIM tended to create more narrow peaks. However, some bile acids did not separate well in nitrogen so the next parameter that was changed was the gas to helium.

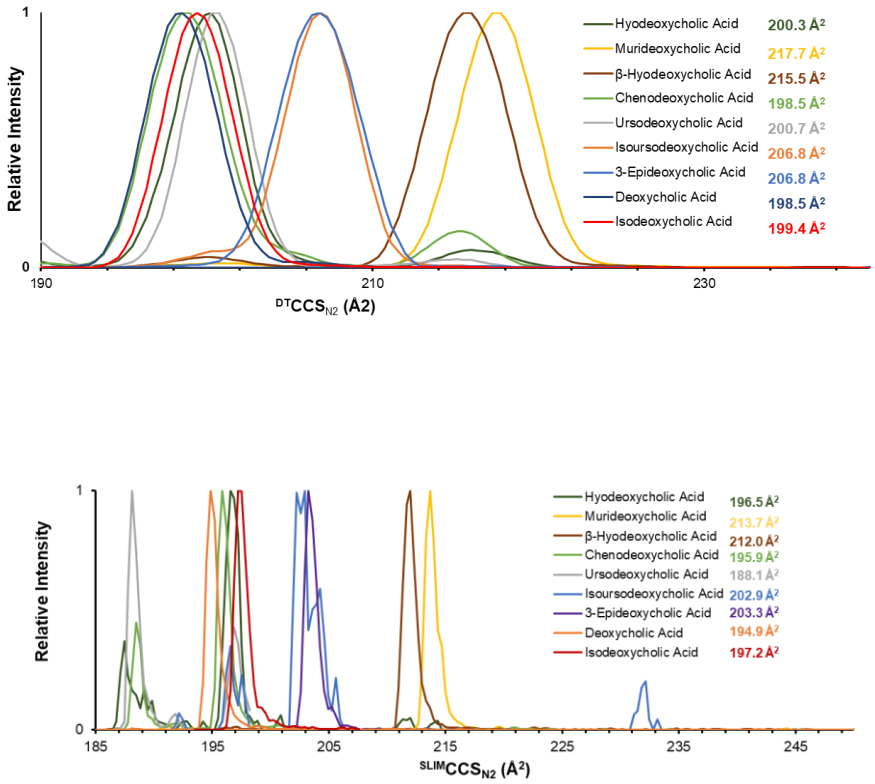



***Figure 2-1:*** *Graphs with different instruments of DT data and SLIM data for isomeric bile acids using* $[M+Na]^+$ *with nitrogen gas.*

Next, with the helium gas, it was expected to have different separation than nitrogen. Since helium is less dense than nitrogen, the ions would travel much faster in the drift tube. This means the CCS values would decrease overall. Additionally, the resolution of the peaks was expected to be sharper because the ions would create less resistance with the lighter gas. In Figure 2-2, a set of bile acids shows with the DT not having any separation and the SLIM showing separation between glychochenodeoxycholic acid and glycohyodeoxycholic acid.

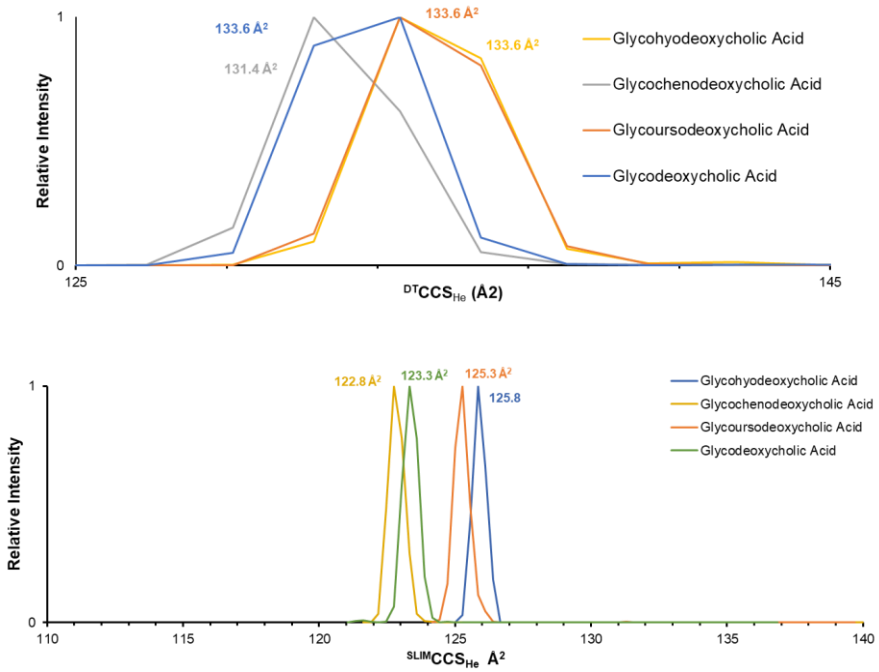



***Figure 2-2:*** *Graphs with different instruments of DT data and SLIM data for isomeric bile acids using* $[M+Na]^+$ *with helium gas.*

The next parameter that was changed during the experiments was the type of mode, whether sodiated or deprotonated. Often for bile acids negative mode works effectively because the molecule ionizes well. However, this experiment showed that the sodiated mode had improved separation and higher resolution. As seen in Figure 2-3, the top graph (deprotonated), demonstrated that the peaks were overlapping, and the CCS values were the same. In contrast, the sodiated peaks on the bottom show less overlap and CCS values have a broader range. Consistently, positive mode showed peaks that were further apart where the CCS values were unique to each compound. Knowing this information can provide insight when performing untargeted metabolomic studies to identify unknown bile acids.

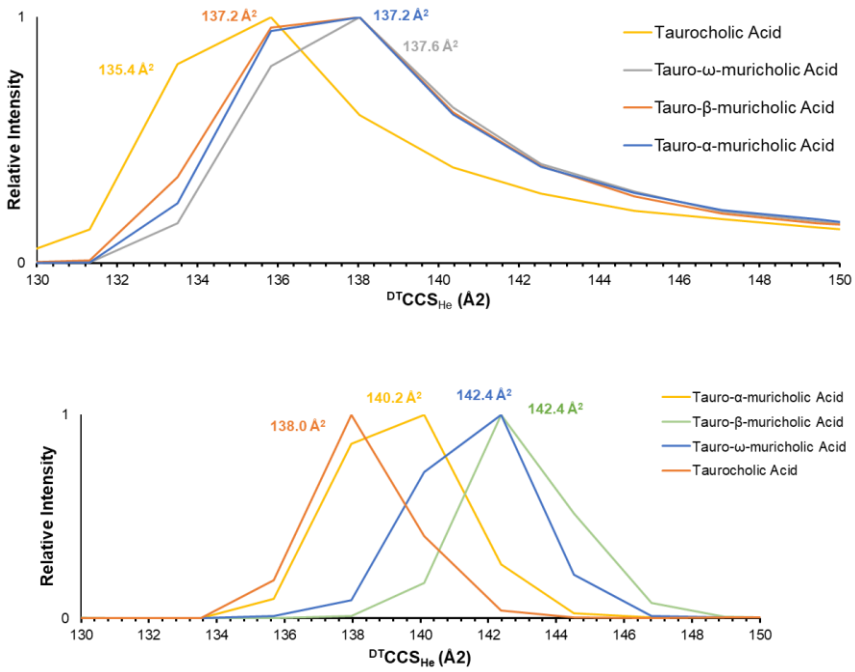



***Figure 2-3:*** *Graphs with different modes of data using DT helium isomeric bile acids. On top is* $[M-H]^-$ *and on bottom is* $[M+Na]^+$.

After the data was collected, the CCS values were calculated and recorded in Tables 2-3 to 2-6. Overall, these tables show the DT and SLIM data are within 2% of each other. The exception is for Table 2-5 which shows the data in positive mode with helium. For helium, the CCS values are outside the percent range because the DT values are generally higher than the SLIM values. This error could be due to a miscalculation in the CCS conversion. Since helium is known to have smaller CCS values, the SLIM CCS values make more sense.

*Table 2-3: Chart of bile acid CCS values in DT and SLIM in positive mode with nitrogen*

| | | $N_2$ Positive Mode | | | |
|---|---|---|---|---|---|
| **Bile Acid** | **Formula** | **$[M+Na]^+$ m/z** | **$^{DT}CCS_{N2}$ (Å2)** | **$^{SLIM}CCS_{N2}$ (Å2)** | **ΔCCS %** |
| Norhyodeoxycholic Acid | $C_{23}H_{38}O_4$ | 401.3 | 204.5 | 181.7 | -12.55 |
| Norursodeoxycholic Acid | | | 201.0 | 185.6 | -8.29 |
| Nordeoxycholic Acid | | | 200.5 | 199.9 | -0.30 |
| Hyodeoxycholic Acid | $C_{24}H_{40}O_4$ | 415.3 | 200.3 | 196.5 | -1.93 |
| Murideoxycholic Acid | | | 217.7 | 213.7 | -1.87 |
| β-Hyodeoxycholic Acid | | | 215.5 | 212.0 | -1.65 |
| Chenodeoxycholic Acid | | | 198.5 | 195.9 | -1.33 |
| Ursodeoxycholic Acid | | | 200.7 | 188.1 | -6.70 |
| Isoursodeoxycholic Acid | | | 206.8 | 202.9 | -1.92 |
| 3-Epideoxycholic Acid | | | 206.8 | 203.3 | -1.72 |
| Deoxycholic Acid | | | 198.5 | 194.9 | -1.85 |
| Isodeoxycholic Acid | | | 199.4 | 197.2 | -1.12 |
| α-Muricholic Acid | $C_{24}H_{40}O_5$ | 431.3 | 214.8 | 212.3 | -1.18 |
| β-Muricholic Acid | | | 217.0 | 213.3 | -1.73 |
| ω-Muricholic Acid | | | 219.6 | 216.7 | -1.34 |
| γ-Muricholic Acid | | | 210.5 | 207.6 | -1.40 |
| Cholic Acid | | | 197.4 | 193.9 | -1.81 |
| Allocholic Acid | | | 203.5 | 201.2 | -1.14 |
| 3β-Cholic Acid | | | 203.1 | 201.2 | -0.94 |
| Ursocholic Acid | | | 200.9 | 197.2 | -1.88 |
| Glycohyodeoxycholic Acid | $C_{26}H_{43}NO_5$ | 472.6 | 207.3 | 203.9 | -1.67 |
| Glycochenodeoxycholic Acid | | | 203.4 | 200.2 | -1.60 |
| Glycoursodeoxycholic Acid | | | 207.8 | 205.3 | -1.22 |
| Glycodeoxycholic Acid | | | 203.4 | 201.2 | -1.09 |
| Glycohyocholic Acid | $C_{26}H_{43}NO_6$ | 488.6 | 207.1 | 203.9 | -1.57 |
| Glycine-β-muricholic Acid | | | 209.7 | 207.6 | -1.01 |
| Taurohyodeoxycholic Acid | $C_{26}H_{45}NO_6S$ | 522.7 | 214.9 | 213.2 | -0.80 |
| Tauro-α-muricholic Acid | $C_{26}H_{44}NO_7S$ | 560.7 | 212.7 | 217.4 | 2.16 |
| Tauro-β-muricholic Acid | | | 217.4 | 219.4 | 0.91 |
| Tauro-ω-muricholic Acid | | | 217.0 | 214.0 | -1.40 |
| Taurocholic Acid | | | 211.3 | 214.0 | 1.26 |

***Table 2-4**: Chart of bile acid CCS values in DT and SLIM in negative mode with nitrogen*

| | | $N_2$ Negative Mode | | | |
|---|---|---|---|---|---|
| **Bile Acid** | **Formula** | **$[M-H]^-$ m/z** | **$^{DT}CCS_{N2}$ (Å2)** | **$^{SLIM}CCS_{N2}$ (Å2)** | **ΔCCS %** |
| Norhyodeoxycholic Acid | $C_{23}H_{38}O_4$ | 377.3 | 209.4 | 207 | -1.16 |
| Norursodeoxycholic Acid | | | 207.9 | 205.9 | -0.97 |
| Nordeoxycholic Acid | | | 201.5 | 199.3 | -1.10 |
| Hyodeoxycholic Acid | $C_{24}H_{40}O_4$ | 391.3 | 203.6 | 208.6 | 2.40 |
| Murideoxycholic Acid | | | 205.2 | 208.8 | 1.72 |
| β-Hyodeoxycholic Acid | | | 212.2 | 208.6 | -1.73 |
| Chenodeoxycholic Acid | | | 205.3 | 207.4 | 1.01 |
| Ursodeoxycholic Acid | | | 203.4 | 206.8 | 1.64 |
| Isoursodeoxycholic Acid | | | 210.2 | 206.8 | -1.64 |
| 3-Epideoxycholic Acid | | | 204.9 | 200.8 | -2.04 |
| Deoxycholic Acid | | | 204.9 | 201.1 | -1.89 |
| Isodeoxycholic Acid | | | 202.5 | 199.1 | -1.71 |
| α-Muricholic Acid | $C_{24}H_{40}O_5$ | 407.3 | 206.2 | 208.8 | 1.25 |
| β-Muricholic Acid | | | 204.7 | 208.6 | 1.87 |
| ω-Muricholic Acid | | | 205.4 | 208.6 | 1.53 |
| γ-Muricholic Acid | | | 204.9 | 208 | 1.49 |
| Cholic Acid | | | 204.9 | 202 | -1.44 |
| Allocholic Acid | | | 206.3 | 203.1 | -1.58 |
| 3β-Cholic Acid | | | 205.2 | 202 | -1.58 |
| Ursocholic Acid | | | 205.4 | 202.3 | -1.53 |
| Glycohyodeoxycholic Acid | $C_{26}H_{43}NO_5$ | 448.3 | **no peak** | 200.3 | |
| Glycochenodeoxycholic Acid | | | 202.4 | 199.7 | -1.35 |
| Glycoursodeoxycholic Acid | | | 203 | 200 | -1.50 |
| Glycodeoxycholic Acid | | | 201.7 | 198.6 | -1.56 |
| Glycohyocholic Acid | $C_{26}H_{43}NO_6$ | 464.3 | 204.1 | 201.1 | -1.49 |
| Glycine-β-muricholic Acid | | | 204.5 | 201.7 | -1.39 |
| Taurohyodeoxycholic Acid | $C_{26}H_{45}NO_6S$ | 498.3 | 210.6 | 207.4 | -1.54 |
| Tauro-α-muricholic Acid | $C_{26}H_{44}NO_7S$ | 514.3 | 212 | 208.6 | -1.63 |
| Tauro-β-muricholic Acid | | | 211.6 | 208.6 | -1.44 |
| Tauro-ω-muricholic Acid | | | 211.9 | 208.8 | -1.48 |
| Taurocholic Acid | | | 210.2 | 206.8 | -1.64 |

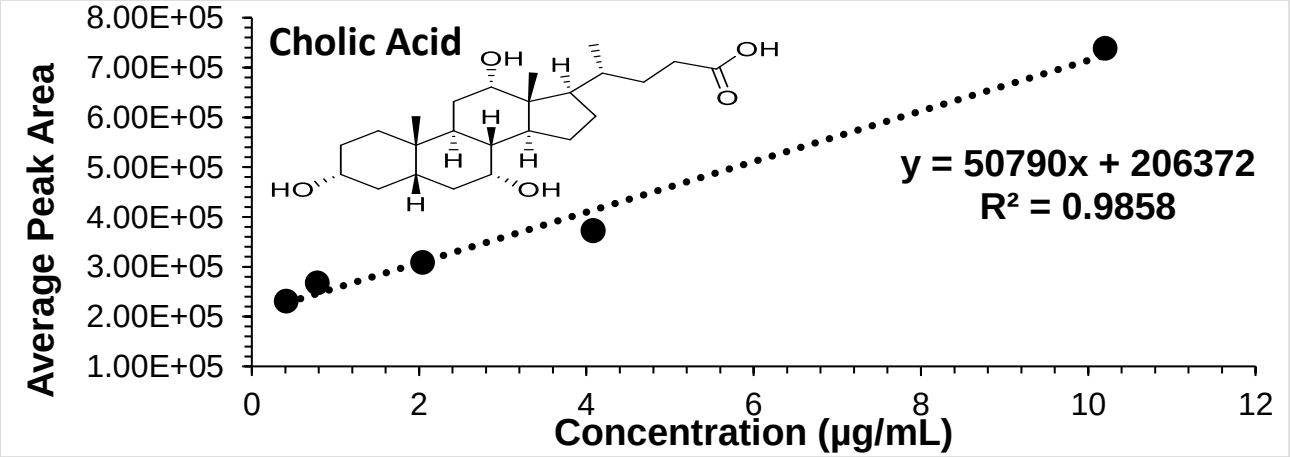
***Table 2-5:*** *Chart of bile acid CCS values in DT and SLIM in positive mode with helium*

| | | He Positive Mode | | | |
|---|---|---|---|---|---|
| **Bile Acid** | **Formula** | **$[M+Na]^+$ m/z** | **$^{DT}CCS_{He}$ (Å2)** | **$^{SLIM}CCS_{He}$ (Å2)** | **ΔCCS %** |
| Norhyodeoxycholic Acid | $C_{23}H_{38}O_4$ | 401.3 | 129.3 | 133.1 | 2.85 |
| Norursodeoxycholic Acid | | | 127.0 | 129.8 | 2.11 |
| Nordeoxycholic Acid | | | 127.0 | 131.1 | 3.09 |
| Hyodeoxycholic Acid | $C_{24}H_{40}O_4$ | 415.3 | 127.0 | 119.1 | -6.63 |
| Murideoxycholic Acid | | | 135.9 | 126.5 | -7.41 |
| β-Hyodeoxycholic Acid | | | 135.9 | 126.2 | -7.67 |
| Chenodeoxycholic Acid | | | 129.3 | 119.1 | -8.55 |
| Ursodeoxycholic Acid | | | 127.0 | 118.2 | -7.44 |
| Isoursodeoxycholic Acid | | | 132.2 | 122.3 | -8.10 |
| 3-Epideoxycholic Acid | | | 131.4 | 121.4 | -8.27 |
| Deoxycholic Acid | | | 127.0 | 117.7 | -7.90 |
| Isodeoxycholic Acid | | | 127.0 | 118.5 | -7.17 |
| α-Muricholic Acid | $C_{24}H_{40}O_5$ | 431.3 | 135.9 | 125.9 | -7.91 |
| β-Muricholic Acid | | | 138.1 | 126.5 | -9.20 |
| ω-Muricholic Acid | | | 140.3 | 128.1 | -9.52 |
| γ-Muricholic Acid | | | 135.9 | 124.5 | -9.12 |
| Cholic Acid | | | 127.0 | 116.2 | -9.27 |
| Allocholic Acid | | | 131.4 | 121.1 | -8.52 |
| 3β-Cholic Acid | | | 131.4 | 120.2 | -9.33 |
| Ursocholic Acid | | | 129.3 | 118.5 | -9.08 |
| Glycohyodeoxycholic Acid | $C_{26}H_{43}NO_5$ | 472.6 | 133.6 | 125.8 | -6.24 |
| Glycochenodeoxycholic Acid | | | 131.4 | 122.8 | -6.97 |
| Glycoursodeoxycholic Acid | | | 133.6 | 125.3 | -6.66 |
| Glycodeoxycholic Acid | | | 133.6 | 123.3 | -8.39 |
| Glycohyocholic Acid | $C_{26}H_{43}NO_6$ | 488.6 | 135.8 | 125.8 | -7.93 |
| Glycine-β-muricholic Acid | | | 135.8 | 126.9 | -7.00 |
| Taurohyodeoxycholic Acid | $C_{26}H_{45}NO_6S$ | 522.7 | 140.2 | 139.2 | -0.67 |
| Tauro-α-muricholic Acid | $C_{26}H_{44}NO_7S$ | 560.7 | 140.2 | 134.4 | -4.29 |
| Tauro-β-muricholic Acid | | | 142.4 | 135.7 | -4.97 |
| Tauro-ω-muricholic Acid | | | 142.4 | 130.6 | -9.07 |
| Taurocholic Acid | | | 138.0 | 130.0 | -6.16 |

***Table 2-6:*** *Chart of bile acid CCS values in DT and SLIM in negative mode with helium*

| Bile Acid | Formula | He Negative Mode [M-H]⁻ m/z | $^{DT}CCS_{He}$ (Å2) | $^{SLIM}CCS_{He}$ (Å2) | ΔCCS % |
|---|---|---|---|---|---|
| Norhyodeoxycholic Acid | $C_{23}H_{38}O_4$ | 377.3 | 126.8 | 121.9 | -4.01 |
| Norursodeoxycholic Acid | | | 126.8 | 121.3 | -4.54 |
| Nordeoxycholic Acid | | | 122.4 | 119.7 | -2.28 |
| Hyodeoxycholic Acid | $C_{24}H_{40}O_4$ | 391.3 | 129.2 | 128.5 | -0.53 |
| Murideoxycholic Acid | | | 130.1 | 128.1 | -1.56 |
| β-Hyodeoxycholic Acid | | | 131.3 | 130.8 | -0.41 |
| Chenodeoxycholic Acid | | | 129.2 | 128.9 | -0.25 |
| Ursodeoxycholic Acid | | | 123.5 | 127.7 | 3.32 |
| Isoursodeoxycholic Acid | | | 130.3 | 129.2 | -0.87 |
| 3-Epideoxycholic Acid | | | 127.4 | 123.7 | -3.03 |
| Deoxycholic Acid | | | 125.7 | 124.1 | -1.26 |
| Isodeoxycholic Acid | | | 124.6 | 122.5 | -1.72 |
| α-Muricholic Acid | $C_{24}H_{40}O_5$ | 407.3 | 129.5 | 130.0 | 0.40 |
| β-Muricholic Acid | | | 129.1 | 128.4 | -0.55 |
| ω-Muricholic Acid | | | 128.3 | 129.2 | 0.67 |
| γ-Muricholic Acid | | | 129.1 | 130.0 | 0.68 |
| Cholic Acid | | | 126.5 | 125.3 | -1.00 |
| Allocholic Acid | | | 128.8 | 125.7 | -2.46 |
| 3β-Cholic Acid | | | 126.9 | 125.3 | -1.29 |
| Ursocholic Acid | | | 128.0 | 125.7 | -1.80 |
| Glycohyodeoxycholic Acid | $C_{26}H_{43}NO_5$ | 448.3 | 129.1 | 127.6 | -1.14 |
| Glycochenodeoxycholic Acid | | | 128.9 | 127.2 | -1.35 |
| Glycoursodeoxycholic Acid | | | 129.1 | 127.2 | -1.46 |
| Glycodeoxycholic Acid | | | 126.9 | 125.6 | -1.00 |
| Glycohyocholic Acid | $C_{26}H_{43}NO_6$ | 464.3 | 129.4 | 127.6 | -1.37 |
| Glycine-β-muricholic Acid | | | 129.0 | 127.2 | -1.44 |
| Taurohyodeoxycholic Acid | $C_{26}H_{45}NO_6S$ | 498.3 | 135.9 | 133.3 | -1.92 |
| Tauro-α-muricholic Acid | $C_{26}H_{44}NO_7S$ | 514.3 | 137.2 | 133.3 | -2.93 |
| Tauro-β-muricholic Acid | | | 137.2 | 133.3 | -2.93 |
| Tauro-ω-muricholic Acid | | | 137.6 | 134.1 | -2.62 |
| Taurocholic Acid | | | 135.4 | 131.8 | -2.75 |

For the final experiment, a series of bile acids were tested for their concentration and compared to the ARUP laboratory test. The bile acids were tested in the SLIM because it has higher resolution than the DT. The results indicated that the SLIM could detect lower limit of detections (LODs) than the laboratory test. The lowest concentration tested was .04 µg/mL which was detected in the SLIM method. According to the laboratory tests, the reference for the concentration of cholic acid was only 0.78 µg/mL. Deoxycholic acid and ursodeoxycholic acid were studied, but their graph was not a linear line which could be due to many factors such as sample contamination or error in the calculation. In Figure 2-4, the cholic acid and chenodeoxycholic acid show a strong trend line to indicate that their concentrations were accurately detected. This equation can be used to find an unknown concentration of bile acids for this test at a specific chromatographic peak area. When performing this experiment with samples in different matrixes (blood, serum, etc.), the method will have to consider extraction methods so there will not be interference from other substances in the results. This method proved to have better sensitivity than the laboratory test and has potential in many clinical settings for bile acid related diseases.



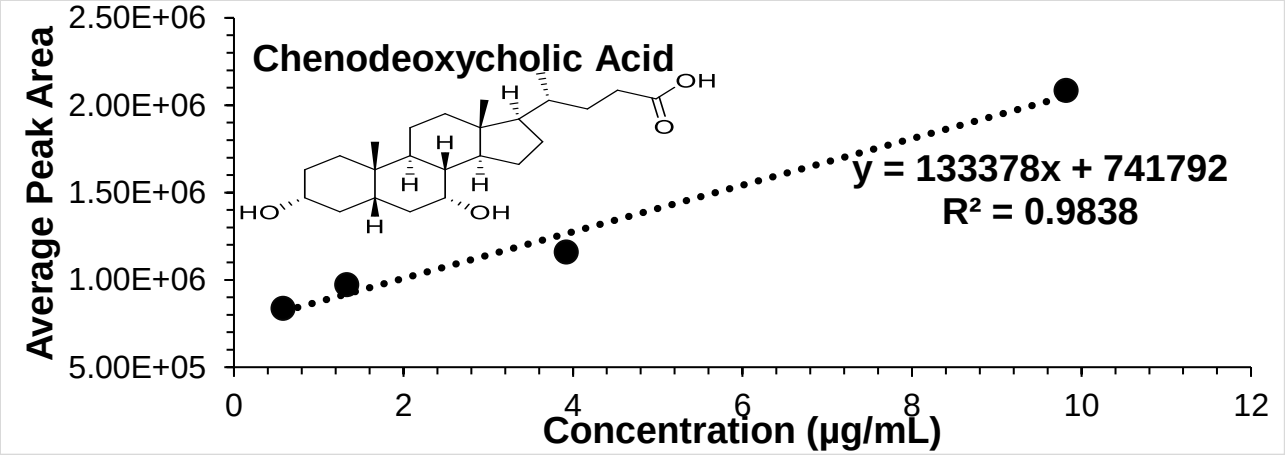



***Figure 2-4:*** *Calibration curves of cholic acid and chenodeoxycholic acid with concentration and average peak area*

## CHAPTER 3: CONCLUSION

Since the bile acids were successfully detected, the goal is to make this method accessible to doctors and clinical professionals. It is important to use this research in clinical settings so that patients can be treated more quickly and effectively. Doctors need to be informed about the benefits of SLIM in clinical diagnostics because it can detect molecules such as isomers that other methods cannot. SLIM has higher resolution and can have results within seconds for physicians to correctly diagnose their patient's diseases. Because patients can be diagnosed faster and more accurately with ion mobility techniques, there needs to be more awareness and education to health care professionals. In the future, ion mobility will be implemented in clinical settings and more communication will be made to inform doctors about the best and most advanced methods to diagnose their patients.

Not only do doctors need to know about ion mobility, but people suffering with digestive issues need to know about their diagnosis. As doctors become informed about

these new analytical techniques, they will communicate confidently to their patients the right diagnosis. People who have rare diseases will finally have answers to their questions and will be relieved to know why they have suffered. Additionally, more drugs and treatments will be developed from this research to cure their diseases. Bile acid metabolism can be the key to avoiding nasty digestive issues and can help people function in regular activities. More research using ion mobility will be necessary to discover biomarkers that will be targeted to treat patients. This research will be a tool to uncover more ideas that can further enhance the clinical world of patient diagnostics. The next projects in this series will be to further analyze bile acids in clinical samples for practical applications.

For a future project, the goal is to conduct an experiment to evaluate patients in a clinical setting affected by liver disease. Since the previous project found the limit of detection of these specific bile acids, those numbers can be applied to study bile acid concentrations in patients' samples. This method will be applied to perform a clinical study on patients with and without liver disease. Patients who are willing to participate will have a liver test performed to see if they qualify as a liver diseased patient or a healthy patient. Then a urine sample will be collected from the patients to be used in the experiment. The samples will be delivered to the lab and stored properly to ensure biomedical safety.

The samples will be extracted through solid/liquid phase extraction to remove any impurities and then prepared for injection in the SLIM. The previous method will be applied using SLIM and the appropriate drift gas and positive and negative mode. Additionally, the data will be processed to find the CCS values and analyzed to

determine the concentrations of the four bile acids detected previously. Further calculations will be performed for statistical analysis and visual representation of the data using principal component analysis (PCA). The healthy and unhealthy samples will be graphed with the concentrations of each bile acid. This type of analysis will be able to compare all the bile acids on one data set to determine their relationship to liver disease. Ultimately, predicting the harmful levels of bile acids linked to liver disease will make for more faster and reliable diagnoses in the future. Clinical studies like this will be important to target the bile acid concentrations related to other diseases, like colon cancer and diabetes. More studies can be performed to analyze certain populations to find out which demographics are more susceptible to the diseases. Once these tests are performed, patients can understand the cause and recognize preventative measures to take for their disease. Additional research will be done with these results to improve biomarker research and create drugs and therapeutic treatments.

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