UNIVERSITY OF CALIFORNIA SAN DIEGO

Methods of High-Throughput Analysis of Cyanobacteria-Derived Succinic Acid

A Thesis submitted in partial satisfaction of the requirements
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by

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The Thesis of Berk Kuntasal is approved, and it is acceptable in quality and form for publication on microfilm and electronically.

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ABSTRACT OF THE THESIS

Methods of High-Throughput Analysis of Cyanobacteria-Derived Succinic Acid

by

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Master of Science in Chemistry

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Professor Robert S. Pomeroy, Chair

High throughput methods are of great interest to multi-billion-dollar industries. High throughput methods can make screening and analysis for assays/experiments efficient and cost-effective. This is especially true for biotechnology or pharmaceutical industries trying to screen for specific analytes of interest produced through genetically modified microorganisms. For this thesis, succinic acid standards in cyanobacteria media were screened using four different instruments. Multiple instruments were used to compare which could be used for high throughput screening for succinic acid. The standards were made in a BG-11, a cyanobacteria growth medium,

to simulate as close to an actual sample that could be received. The instruments used for this experiment were HPLC, GCMS, HSGC, and CIMS. Fisher esterification was performed on the succinic acid standards to be analyzed on the GCMS, HSGC, and CIMS. The esterification was conducted not through reflux but by placing the reaction mixture in air-tight crimp vials and placing the vials in a hot water bath. The duty cycles to screen succinic acid per sample through HPLC, GCMS, HSGC, and CIMS were approximately 17min, 5.93min, 5.25min, 3min, respectively.

INTRODUCTION

Purpose of the Experiment

The use of microorganisms has been a staple research topic in the biotechnology and pharmaceutical industries. These microorganisms can range from unicellular to multicellular, with each type having distinct uses. One of the popular uses of microorganisms is the wide range of metabolites they can offer [Putatunda, 1]. These metabolites can either be primary or secondary [Kumar, 2]. Secondary metabolites do participate in the growth and development of the microorganism but are instead produced by the microbe depending on its interaction with its environment [Kumar; Stewart, 2,3]. From secondary metabolites, many natural products that are known today, such as antibiotics and anticancer agents, can be acquired [Kumar; Stewart, 1- 3]. Primary metabolites, such as enzymes and carbohydrates, are key to the microorganism's development and growth [Kumar, 2]. In fact, of the 4000 enzymes that are known, about 200 of them are used commercially, with a worldwide sale worth $3.74 billion [Liu, 4]. However, not every microorganism can produce a metabolite that can be used to make antibiotics, biofuels, biopesticides, etc. Even when the microorganism that produces the desired metabolite is discovered, not every strain of the microorganism produces the same quantity. Since there can be this type of variability on the microorganism types and the quantity of the metabolite production, screening assays and procedures are devised and executed to find the microorganism that produces the metabolite of interest.

Researchers and lab technicians are required to perform numerous experiments and assays. The procedure for these experiments and assays usually contains multiple steps to prepare one sample. Usually, the said samples are prepared in a 96-well plate style. So, in a day's work, the lab technician can prepare up to 100 samples, which need to be screened and analyzed for biological

activity or the analyte of interest. Combining the time spent on sample preparation and the wait time for the screening and analysis, these multi-billion-dollar companies would want to optimize the productivity of their lab technicians to be as cost-effective as possible. Increased efficiency demands a high throughput method of screening and analysis. This paper aimed to develop a high throughput method using HPLC, GCMS, HSGC, and CIMS to screen and analyze cyanobacteria secreted succinic acid.

What is Succinic Acid and its Uses in the Real World

In recent years, a growing push has been against the industrial use of petrochemicals and fossil fuels. When fossil fuels are burned, they omit CO2, which is a known greenhouse gas and causes what is known as the greenhouse effect. Atmospheric carbon dioxide is one of the leading causes of global climate change on Earth. Economic pressure from increasing gasoline prices and the depletion of fossil fuel reserves is another leading reason to look for alternative, more sustainable sources. Significant research and development time has been allocated to discover the use of renewable resources to replace fossil fuel usage. One such method is to convert food waste into bio-based chemicals and energy using microorganism bioprocesses, such as fermentation and photosynthesis [Merrylin, 5]. Another renewable source uses non-food-based materials, such as lignocellulosic biomass [Lan, 6].

Succinic acid was one of those chemical compounds that was initially made using petroleum. It was made using catalytic hydrogenation of maleic acid or maleic anhydride derived from petroleum [6]. Succinic acid, also known as butanedioic acid, is a dicarboxylic acid with four carbons and is an odorless, white crystal at room temperature [Saxena, Beauprez; 5-8]. The chemical structure of succinic acid can be observed in **Figure 1** below.

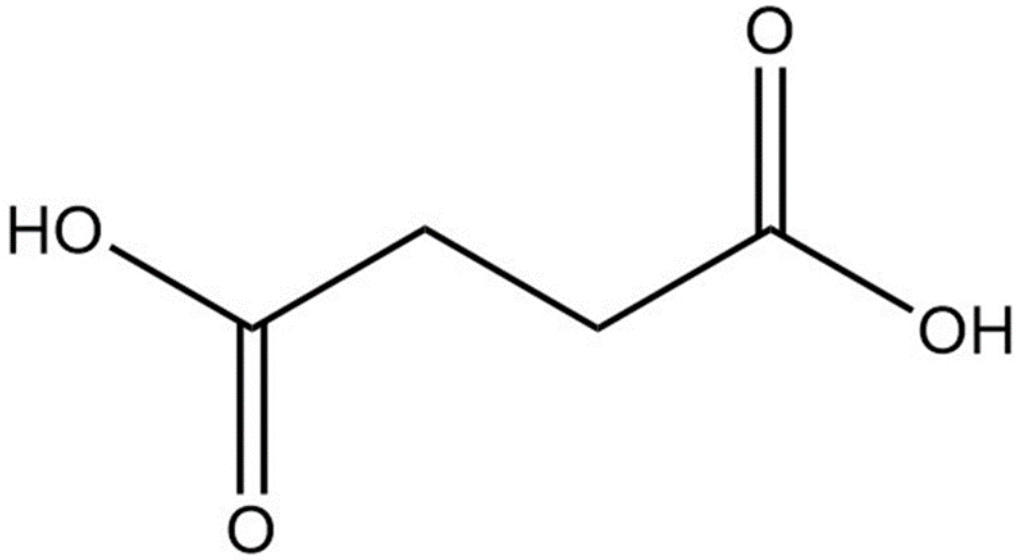


Figure 1: Chemical structure of succinic acid.

It was initially known as amber acid, where the external layer of amber contained succinic acid and was used as an antibiotic in Europe [7]. Today, succinic acid can be used to derive other compounds that are used in food ingredients and additives, detergents/surfactants, plant growth stimulants, fungicides, ion chelators, commodity chemicals, and pharmaceuticals [5-8]. The succinic acid market is so enormous that the yearly production is approximately 20,000 to 50,000 tons [6, 8]. This large-scale production of succinic acid led the U.S. Department of Energy to list succinic acid as one of the top ten high-value added biologically processed chemicals [6-8]. Many commodity chemicals can be derived from succinic acid. There are pyrrolidones, which are mainly used in solvent and polymer industries [8]. There is 1,4-butanediol (BDO), which is used in making polymers, tetrahydrofuran derivatives (THF), and gammabutyrolactone derivates [6,8]. Succinic acid can also be used to derive THF itself, a common solvent used in organic chemistry [6,8].

What is Polyurethane and its Chemical Composition

Polyurethanes are a type of polymer that has many uses in automotive, industrial, and even medical fields [Howard, 9]. The reason for this is because of the versatility polyurethanes have in their many physical, thermal, chemical, and mechanical properties [Zia, Shelke; 10, 11]. Polyurethanes are used in various applications like insulation, furniture, roofing, coatings, adhesives, fibers, paints, and elastomers [Das; 9, 10, 12]. A simple chemical structure of polyurethane is shown in **Figure 2** below.

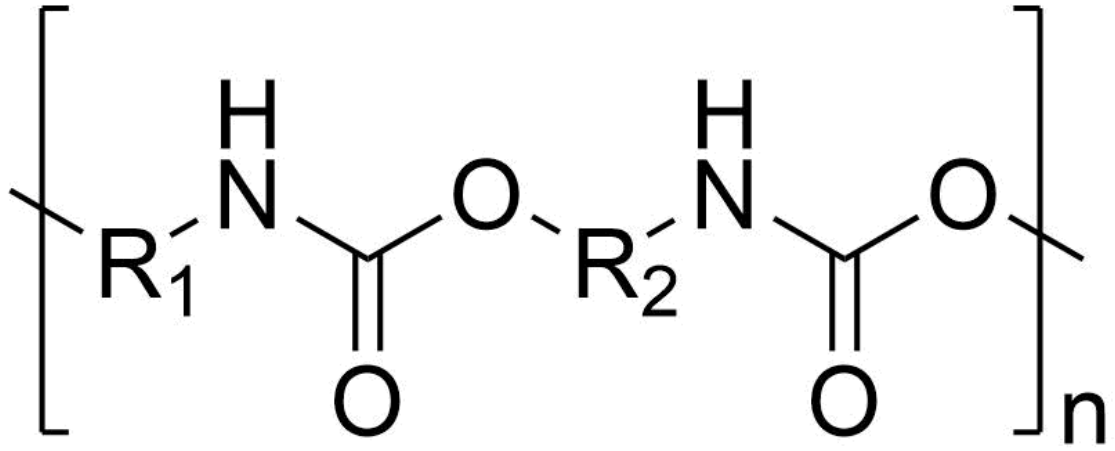


Figure 2: Simplified chemical structure of polyurethane.

Polyurethanes can be achieved through the reaction of isocyanates and polyols [9-12]. **Figure 3** below shows a simplified chemical reaction equation between a polyol and isocyanate.

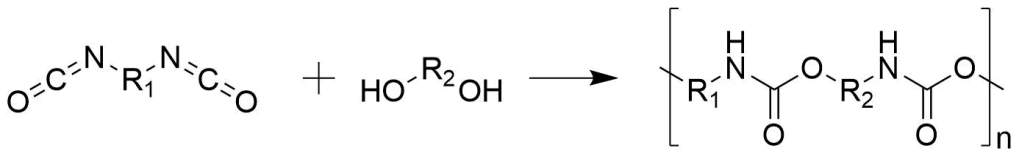


Figure 3: Chemical reaction between a polyol and an isocyanate to form a polyurethane chain.

The reaction between polyols and isocyanates ends up forming different regions in polyurethane structure, known as hard and soft segments [9, 11-12]. These hard and soft segments give polyurethanes their versatility by being rigid, soft, or flexible when the two chemical ratios are altered [9, 11-12]. The polyols provide softness and flexibility, which contain longer chain lengths in the structure and are of low molecular weight [Touchet; 12-13]. The rigidness and hardness provided by the isocyanates, accompanied by chain extenders, are short-chain compounds that show crystallization and high glass transition temperature, making the segment more compact and more rigid [12-13].

How is Succinic Acid Converted to Polyols and its Relation in Making Polyurethanes

Originally, polyols and isocyanates were derived from petrochemicals; however, due to the concerns that were discussed earlier, it was discovered that polyols, isocyanates, and the chain extenders of polyurethanes can now be produced through renewable sources [Zhang, Lim; 14, 15]. One such method is using biologically produced succinic acid and converting it to 1,4-butanediol. The 1,4-butanediol is used as a chain extender in the polyurethane synthesis [11]. The succinic acid can be converted to diol through hydrogenation using bimetallic catalysts, such as Pd-Re and Pt-Re [Kang, 16].

The two most common types of polyurethanes are the ones that are made from polyether or polyester diols as the soft segments [11-12]. Both polyester and polyether polyurethanes have their benefits and weaknesses. Polyurethanes that are formed with polyester polyols as the soft segments usually give the plastic more strength and hardness [12]. This is provided by the fact that polyester polyols have enough hydrogen bonding to provide strong bonds [12]. Polyester polyols

can be formed from raw materials, such as petroleum, or through reclaimed raw material [12]. These raw materials are then made into glycols and diacids, such as succinic acid, which can then be made to produce polyester polyols [12]. The raw materials are formed into polyester polyols through poly-esterification and the reclaimed can be converted through transesterification [12]. Polyester polyols also provide flame retardance and oxidative stability, however, they are usually more viscous and are more expensive than polyether polyols [12]. Polyether polyols are usually formed through polymerizing epoxy monomers or through ring opening reactions [12]. Polyether polyols provide polyurethane with flexibility and hydrolytic stability [12-13]. They are also usually less expensive than polyester polyols and are less viscous [12-13]. For these reasons, they are usually the ones that are used to make polyurethane plastics commercially. However, they do not have much oxidative stability and are flammable [12].

When it comes to environmental benefits, however, polyester based-polyurethanes are more suitable than polyether-based polyurethanes. Polyesters polyols provide polyurethanes with the ability to be hydrolyzed [12-13]. This would mean that some species of microorganisms, such as bacteria and fungi, can hydrolyze the ester bonds in the polyurethane, allowing for biodegradability to occur [9]. It is said in one paper that these microorganisms can hydrolyze the ester bonds in the polyurethane using enzymes, such as esterase and/or urease [9]. The ability to biodegrade gives polyester polyurethanes to be engineered into implants and drug delivery systems [11].

What are Cyanobacteria and its Uses in the Real World

Cyanobacteria are a specific group of photosynthetic prokaryotic microorganisms [Vincent, Singh, Abed; 17-19]. There are approximately 2,000 species of cyanobacteria, and

although they all go through oxygenic photosynthesis, certain species use anoxygenic photosynthesis [17, 19]. Cyanobacteria are also called blue-green algae because of the chlorophyll (blue-green), phycobiliproteins phycocyanin (blue), and the fact that they have an algae-like structure [17]. Cyanobacteria possess significant adaptation mechanisms enabling them to thrive in their environments, be it temperature, photo-oxidation, or osmotic stress [Nowruzi, 20]. They are resilient enough to thrive under minimum water, light, and carbon dioxide [18]. For these reasons, cyanobacteria are an excellent contender for research in multiple different applications in different industries [19]. They are used in wastewater treatment centers because of their ability to bioremediate some heavy metals, increase oxygen concentration in the water through photosynthesis, and are cost-effective methods to use [Ahmad; 18, 21]. They can degrade multiple toxic compounds, protect crops from pathogens, and fix atmospheric nitrogen [18].

How Cyanobacteria Produce Succinic Acid and its Genetic Modification to Produce More

Cyanobacteria can also be used to produce natural products [20]. These natural products can be obtained by performing metabolic engineering on the cyanobacteria [Treece; 6, 22]. Metabolic engineering is manipulating genetic components of a microorganism's chromosome or a plasmid by deleting, replacing, or inserting different genes to push the metabolic process toward the desired compound [Cho, 23]. In industry, this type of genetic manipulation of a microorganism to produce natural products through renewable resources is called microbial cell factories [23]. Many natural products, such as diols, olefins, and organic acids, can be obtained through the metabolic engineering of cyanobacteria [6]. However, for this paper, the discussed chemical will be succinic acid. Succinic acid, or succinate, is a naturally occurring chemical and a crucial intermediate compound in the general Kreb Cycle (also known as the Citric Acid or Tricarboxylic

Acid Cycle) [6, 22]. **Figure 4** below shows an example of the Kreb Cycle. However, most cyanobacteria lack the complete Kreb Cycle, and usually, the cycle is branched out into left and right pathways [6, 22].

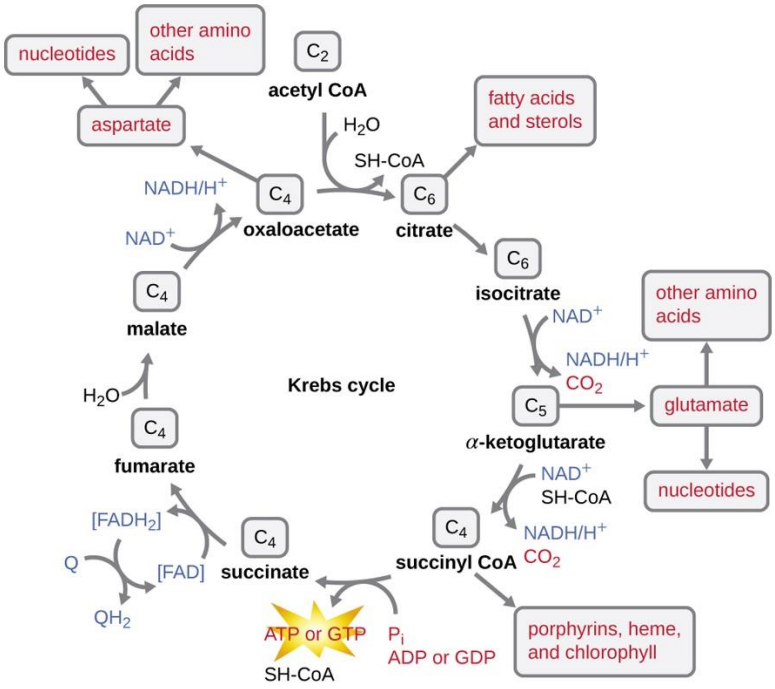


Figure 4: The image shows an example of the Kreb Cycle. This image was obtained through CNX OpenStax, CC BY 4.0 <https://creativecommons.org/licenses/by/4.0>, via Wikimedia Commons: https://commons.wikimedia.org/wiki/File:OSC_Microbio_08_02_KrebsUsage.jpg. The original source can be found in: https://cnx.org/contents/5CvTdmJL@4.4

The reason is that most cyanobacteria do not have the enzymes that convert α-ketoglutarate to succinate [6, 22]. A strain of cyanobacteria, PCC 7002, was discovered to produce succinate by having succinate semialdehyde as an intermediate [6, 22]. The genes that allowed this pathway from PCC 7002 were introduced to a strain of cyanobacteria, PCC 7942 [6, 22]. The PCC 7942 strain was chosen because this strain could also be engineered to be photomixotrophic [6, 22]. This modification allows the photosynthetic strain to use sugar (glucose) to proceed with its metabolic

process [22]. It should also be noted that the strain, PCC 7942, could still use carbon dioxide in its metabolic process [22]. Using the genes from PCC 7002 and the genetic manipulation to make PCC 7942 photomixotrophic, PCC 7942 could produce succinate by using glucose obtained from waste biomass through lignocellulosic hydrolysis and carbon dioxide [22]. Naturally, cyanobacteria do not like glucose as an intake [22]. So, to bypass this, one proton symporter was used to facilitate the import of glucose and another to export the succinate to the media [22]. The import of glucose required to be performed at a low to neutral pH, and the succinate export required a high pH [22] by deleting the gene sdhB (succinate dehydrogenase subunit B) [22], ensured that the Kreb Cycle was interrupted and that the cycle stopped at succinate.

What is High Through Screening Method, Why it is Needed, and How it can be Achieved

As the cyanobacteria are genetically engineered to produce and secrete out more of the succinic acid, a screening method is required to pinpoint which of the culture batches of cyanobacteria are producing the succinic acid and which are not. There are, of course, conventional methods of screening for the strain of interest. One of these is called culture-dependent identification techniques, consisting of phenotypic and genotypic methods [Carraro, Temmerman; 1, 24, 25]. The phenotypic method relies on morphological, metabolic, and physiological characterization of the microorganism through in vitro testing [24, 25]. On the other hand, the genotypic methods rely on the concept of polymerase chain reaction (PCR) [25]. The phenotypic methods are usually known to be cheaper when compared to genotypic methods because they require no special equipment to perform the procedures [25]. However, the reproducibility of phenotypic methods could be better and biased, and complex growth conditions can cause incorrect results by the ambiguity of specific techniques [24, 25]. The genotypic methods also have

their limits because many of the methods' procedures require multiple different PCR-based techniques [25]. Overall, both methods of traditional screening for the target microorganism are highly time-consuming, which, in turn, is costly for companies that work on an industrial scale [1].

For the industries with cell factories, the microorganism strains that produce the target chemical versus the ones that do not need to be screened in rapid succession. For this reason, high-throughput screening (HTS) methods were developed. HTS is performed using automated equipment/instruments to screen for hundreds to thousands of samples per day [26-27]. These automation and technological advancements range from robotic assistance in liquid/gas sampling to highly advanced data processing software and sensitive detection techniques [Avery; 26, 28]. Since significantly more samples could be rapidly processed through HTS, the samples could then be prepared and contained in microtiter plates (MTP). These plates were initially created to have 96 wells [Inglese; 26-29]. However, in recent years, the 96-well plate format has been replaced by 384-well (4 times 96-well) and 1536-well (16 times 96-well) plates, allowing for more samples to be prepared [26-29]. It should also be noted that as the number of wells on the plate increased, the volume of samples needed decreased [27]. The volumes required per well between the 96, 384, and 1536-well plates were 100-200µL, 30- 100µL, and 2.5-10µL, respectively [27]. These volumes have a significant advantage over procedures that require glassware/equipment that have a working volume of milliliters, such as test tubes and cuvettes [29]. This decrease in working volume meant that the assays for HTS can be miniaturized [26-29]. This miniaturization led to assay components to be more optimized [28-29]. The HTS assays were now more uncomplicated and straightforward since the working volume had decreased substantially, and the screening was left to the instrument [28-29]. The decreased working volume meant fewer samples and reagents were required for the assay, leading to a more cost-effective procedure [28-29]. The use of

automated instruments, whether the sampler or the detector, meant that most human errors were eliminated with the reproducibility and sensitivity of the assay increasing [28-29]. The detectors for these instruments also have simple readouts, such as absorbance, allowing for quick screening and analysis by a human [28-29].

HPLC Purpose/Uses, Advantages, and Disadvantages

These automated instruments can be used to screen for succinic acid by creating a fast and reliable qualitative method. One such instrument that has been used in the past to screen for succinic acid was High Performance (can also be Pressure) Liquid Chromatography (HPLC). One paper used a Thermo Scientific Ultimate 3000 HPLC with a UV detector at a wavelength of 210nm to screen for succinic acid that was secreted out of a cyanobacteria strain [22]. In the pharmaceutical industry, HPLC can be used to screen and quantify drugs and metabolites that are acquired from human plasma and urine [Nikolin, 30]. Today, many HPLCs are marketed as UHPLCs (Ultra) where the pressure in the system can be approximately high as 20,000psi and the and the packing in the columns is sub-2μm particles [31]. Allowing smaller particle packing and higher pressure allows HPLCs to obtain higher resolution and faster run time. HPLCs can have different methods of analysis depending on the detector, column (effects stationary phase), solvents (effects mobile phase), and run conditions used. For this experiment, succinic acid was screened using reversed-phase chromatography with a fixed wavelength UV detector and a gradient condition. Reversed-phase chromatography is commonly used for small polar compounds [31]. This method fits succinic acid because it has only four carbons and is polar because of the two carboxyl groups. Reversed-phased columns are packed with silica that have hydrophobic functional groups attached to them (usually a carbon chain) and are driven by hydrophobic

interactions [31]. The solvents chosen for reversed-phase are polar solvents, such as water and acetonitrile [31]. The elution time for the analyte of interest depends on the interactions the analyte has between the mobile and stationary phase [31]. The elution times can be altered by varying the polarity of the solvents [31]. This is why a gradient method is preferred over an isocratic method. By varying the concentrations and type of solvents used in the HPLC method, the run times can be shorter and increase the resolution of the peaks [31]. However, this adds some complexity to HPLC methods since different solvents and concentrations must be tested with the column of choice to discover an optimized method to analyze the compound [31]. This means many trials and errors must be conducted between the stationary and mobile phases [31]. The fixed wavelength UV detector gives more sensitivity than a diode array detector by allowing the HPLC to analyze the sample for one wavelength [30-31]. Having a UV detector also means that the samples being analyzed can technically be recovered since UV absorption is not a destructive process. HPLC also allows the user to analyze compounds with masses in the kilodalton (kDa) range. Although the automation of HPLC, and the other instruments that will be discussed in this chapter, allows for more accurate and reproducible results, one disadvantage of automation is the cost of the instrument. Since HPLC is also built to have only liquid running through the system, the solvent and bottle needs to be refilled frequently. If any air bubbles get stuck in the system, then the instrument needs to be shut down and a technician needs to be requested. Resulting in an expensive and time wasted accident.

GCMS Purpose/Uses, Advantages, and Disadvantages

Gas chromatography-mass spectrometer (GCMS) is another analytical instrument that can be used to screen for succinic acid. GCMS can be applied to multiple fields with the purpose of

identifying the compounds and then quantifying them [Sparkman, 32]. Some examples of the fields GCMS is used in are forensics, food industry, health care, biotechnology, and environmental science [32]. One paper showed how GCMS/MS can be used in forensic toxicology laboratory to identify common antidepressants from human blood samples [Truta, 33]. Two other papers used GCMS and GCMS/MS to identify and quantify organic acids, one of which is succinic acid, from human urine samples [Mouskeftara, Christou; 34, 35]. Just like HPLC, the samples that are introduced into the GCMS are diluted in some solvent. Unlike HPLC, the mobile phase of GCMS is a gas and the user usually does not change the gas [31]. The gas chosen for GCMS, or GCs in general, are inert gases, such as helium or argon [31]. So, the mobile phase of a GC is just to carry the sample through the column into the detector [31]. Using an inert gas means that the sample will only interact with the stationary phase. This is an advantage the GCMS has over the HPLC because, unlike the HPLC where the mobile and stationary phase must be considered, mainly the stationary phase needs to be accounted for GCMS. The most used GC is gas-liquid chromatography (GLC) [31]. GLC is the type of GC where the mobile phase is a gas, and the stationary phase is a liquid coating on the walls of the column [31]. Common GC columns are capillary columns, not packed, meaning the columns are open tubular allowing them to be narrower and significantly longer [31]. The increase in the length of the column allows for more efficient separation of compounds [31]. The type of column used in this experiment was a fused-silica wall-coated (FSWC) capillary column. This type of column has a polyimide coating for protection and is flexible, allowing for it to be coiled up, which is necessity since the column length can be from 10-100m [31]. It should be noted that the samples that can be introduced into the GC need to be approximately 500Da, which is smaller than what can be introduced into the HPLC, because the samples need to be volatilized and be sent to the carrier gas. The elution times for the

compounds in the sample can be altered by controlling the temperature in the oven, where the column resides [31]. The adsorption of compounds to silica decreases with increasing temperature. This allows lower boiling point compounds to be eluted earlier and higher ones later [31]. The MS of the GCMS works by ionizing, through electron ionization (EI), and fragmenting the compounds in the sample to a mass-to-charge ratio (m/z) [31]. The MS detector (MSD), unlike the UV detector of HPLC, is destructive, meaning the sample cannot be recovered at the end of the run [31]. However, the MSD is a universal detector that can detect anything if the sample is ionized [31]. The GCMS also arrives with chemical libraries in the software. This library has thousands of chemicals in its database, and it allows the user to compare the m/z ratios, respective to the chromatogram peak, to other chemicals in the library. This way the user can confirm what chemical each peak corresponds to in the chromatogram. The one thing that needs to be cautious about for MSD is to make sure that it does not get overloaded with concentrated samples [31]. This is why the samples need to be diluted and even add a detector delay to the method program, or else the detector can degrade. The GCs also have split injections that help dilute the sample by having some ratio of sample go through the column and the rest to go to the waste [31].

Why Esterification of Succinic Acid is Needed for GCMS

The succinic acid needs to be derived into the ester form because dimethyl succinate (DMS) can be screened and analyzed better through the GCMS. The reason why the succinic acid needs to be esterified is because the ester form has a higher volatility than the carboxylic acid form. For the GC to work properly, the injected sample needs to be able to be volatilized in the GC inlet and introduced into the column in the gas phase. The acid form has a higher boiling point than the ester form. The acid form has a boiling point of 235°C and the ester form has a boiling point of

196°C. The succinic acid will probably thermally degrade before it can vaporize. The reason for such different boiling points is because acids are more polar than esters, so esterification increases volatility by decreasing the polarity of the compound. Succinic acid was derived into DMS through Fischer Esterification. **Figure 5** below shows an example of Fischer Esterification on succinic acid. The esterification was performed by reacting methanol, with an acid catalyst, and succinic acid.

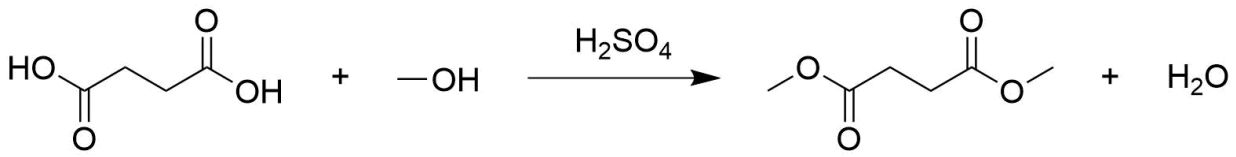


Figure 5: Esterification of succinic acid to DMS using methanol and sulfuric acid as the catalyst.

HSGC Purpose/Uses, Advantages, and Disadvantages

Besides an automated liquid sampler, the GC can also have an automated head space sampler converting the GC to HSGC. HSGC can be used in the same fields as GCMS, such as the food industry and forensics [Yao, Monteiro, Monteiro; 36, 37, 38]. A paper analyzed the volatile chemicals that occurred during the processing stages of Dezhou braised chickens using HSGC-IMS [36]. One paper analyzed how anesthetics can affect human blood alcohol analysis conducted through HSGC [37]. Another paper conducted a qualitative and quantitative test on human blood and urine samples for VOCs [38]. The HSGC samples are prepared in crimp vials that can be sealed airtight by using a special crimp cap with a septum. The headspace in HSGC is the vacant space above the liquid/solid sample in the crimp vial. This empty space is where the volatile species from the sample matrix can be separated using the GC and analyzed by the detector from the non-volatile species by heating the vial in the HS oven. HSGC is handy when the user wants

to identify and analyze specific chemicals that are in the sample matrix but does not want to inject the whole matrix for fear of contaminating the GC. This allows the HSGC to obtain cleaner chromatograms than injecting the liquid sample. HSGC works by reaching an equilibrium within the crimp vial when the sample is heated. However, one of the disadvantages with headspace sampling is that the chemical relationships within the matrix and the gas laws, such as Henry's Law and Raoult's Law, can drastically affect the goal to reach equilibrium. These issues can usually be optimized through multiple trial and error runs of changing parameters such as heating of the vial, volume ratios between the analytes and solvent, vial shaking rate, and sample/standard preparation procedure. The detector that was used for the HSGC was a flame ionization detector (FID). The flame is created by using a mix of air and hydrogen [31]. The carrier gas still needs to be inert, just as the GCMS. In this flame, organic compounds are pyrolyzed into ions and electrons [31]. These ions and electrons carry an electrical charge where they can be detected as microamps or microvolts [31]. FID has its many advantages and disadvantages. Since FID is a mass-sensitive detector, instead of a concentration-sensitive, changes in carrier gas flow rate do not affect the detector response drastically [31]. The FID has small noise, high sensitivity, and has a decent linear response [31]. The FID can also be considered a universal detector, however, there are compounds that do not receive any response from the detector [31]. This can be beneficial or a detriment, depending on the experiment's application. The compounds that do not give a signal in FID are usually non-combustible compounds, such as carbon dioxide and nitrogen oxides [31]. Certain functional groups such as carbonyls, amines, and halogens can only be ionized with the flame very little to none of them ionizing [31]. Just like the MSD, the FID is also a destructive detector, and the samples cannot be recovered at the end of the run [31].

Why Esterification of Succinic Acid is Needed for HSGC

For the HSGC, the succinic acid also needed to be derivatized to its ester form. This reasoning is the same as the discussion had before. The esters have lower boiling points than carboxylic acids. This facilitates the process of getting sufficient analyte into the headspace of the vial. However, for this experiment, it was discovered that, instead of performing the esterification through reflux, the esterification was done inside the HSGC crimp vials.

CIMS Purpose/Uses, Advantages, Disadvantages, and Why is Esterification of Succinic Acid Need for CIMS

Chemical ion mass spectrometer (CIMS) was the last instrument used to analyze succinic acid for this experiment. CIMS can be used in environmental science and forensics [Zhang, Lewis; 39-40]. One paper used CIMS to measure trace gases in the atmosphere [39]. Another paper stated that CIMS can be used to perform drug analysis, such as finding the number and purpose of active hydrogens in morphine [40]. Unlike electron ionization, which is known as hard ionization, chemical ionization is known as soft ionization [31, 40-41]. It is called soft ionization because the collision energy is lower and there are fewer fragments available [31, 40-41]. The CIMS works by having a reagent gas flow through a heated filament/probe that bombards the reagent gas with electrons, like electron ionization, and turns it into reagent ion [31, 40-41]. These reagent ions then collide and react with the injected sample [31, 40-41]. These reactions occur in an ion-molecule reactor (IMR) [31, 40-41]. The benefit of soft ionization is that it allows the user to determine the molecular masses of compounds [31, 40-41]. The most common type of reaction that occurs in CIMS is proton transfer, which is where the proton from the reagent ion is transferred to the analyte

[31, 40-41]. The second type of reaction that can occur is proton abstraction, which is where the reagent ion obtains a proton from the analyte [31, 40-41]. The advantages of CIMS lies with its high selectivity and sensitivity. However, like the HSGC, the analyte in the sample needs to be volatile [31, 40-41]. This is why the succinic acid again needs to be derived to its ester form. The user also needs to be careful with the choice of the reagent gas [31, 40-41]. Although there are options to the reagent gas, each gas gives a different spectrum [31, 40-41]. For this experiment, benzene was used as the reagent gas. The use of benzene as the reagent gas meant that the reaction type in the CIMS was a charge-exchange and the CIMS needed to be placed positive ion mode. The reason for switching to positive mode is because in charge-exchange reaction the benzene would become a radical compound with a positive charge. This radical cation would then be transferred to the analyte of interest, DMS, hence why the CIMS need to be switched to positive mode.

Chapter 1 METHODS

Section 1.1: Fisher Esterification of Succinic Acid

The Fisher Esterification was performed in a 20mL crimp vials with a rubber septa and aluminum crimp caps. The crimp vials were clamped onto a ring stand and lowered down into a recrystallization dish filled with water at approximately 95-100°C. The water in the recrystallization dish was heated using a heating block. The reaction ran for 2.5hrs at mid to high stirring speed. **Figure 6** below shows the glassware setup for the reaction.

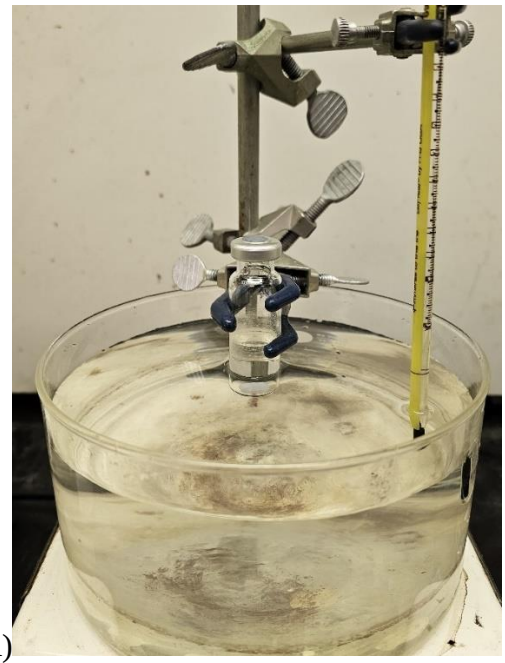
A)  B) 

Figure 6: The glassware setup (image A) for the Fisher Esterification of succinic acid to DMS and the type of crimp vials used (image B).

The solvent used to make succinic acid standards was BG-11, a medium that is used to grow cyanobacteria. The reaction inside the crimp vial was performed by placing 1mL of succinic acid standard, 20 drops of 17.5M sulfuric acid using a glass Pasteur pipette, 9mL of methanol, and

a small stir bar. The ratio inside the solution is approximately 1:11 of succinic acid standard to methanol and sulfuric acid.

Section 1.2: HPLC Parameters and Sample Preparation

An Ultimate 3000 Thermo Scientific HPLC was used to analyze succinic acid. **Figure 7** below shows an image of the HPLC used.

Figure 7: An image of the Ultimate 3000 Thermo Scientific HPLC.

The succinic acid was analyzed using an UV detector at 210nm with an Acclaim^{TM} Polar Advantage II column that was a C18 at 3µm particle size with 2.1 X 150mm dimensions. The succinic acid standard was placed in a 2mL vial. A gradient method with a combination of 0.1%

formic acid buffer in MQ at a pH of 3.35 and methanol was used. The flow rate that was used throughout the method was 0.2mL/min and the method ran for 17min. The injection volume for each sample was 10µL. The succinic acid standard was prepared using BG-11 medium. For each 2mL vial, 500µL of succinic acid standard is added, and then 20µL of 88% pure formic acid added. The formic acid is added to make sure the solution stays acidic. That way, the two carboxylic acids of succinic acid continue to be protonated. **Table 1** below shows the method parameters for the flow gradient used for the analysis.

Table 1: Eluent B is methanol and eluent C is the 0.1% formic acid (FA) buffer. The flow rate is constant at 0.2mL/min throughout the method. The method starts off with 100% FA for 7min. From 7 to 7.5min, the eluent ratio changes to 70% methanol and 30% FA. This eluent mixture is there to wash the column. This ratio is kept until 11min. After, the ratio is changed back to 100% FA for the rest of the run to condition the column back to the starting position.

Time	**Flow (mL/min)**	**%B**	**%C**	**%D**	**Curve**
0.000					
0.000	0.200	0.0	100.0	0.0	5
7.000	0.200	0.0	100.0	0.0	5
7.500	0.200	70.0	30.0	0.0	5
11.000	0.200	70.0	30.0	0.0	5
11.100	0.200	0.0	100.0	0.0	5
17.000					

Section 1.3: GCMS Parameters and Sample Preparation

The GCMS analysis of DMS was performed on an Agilent 7820A GC system with an Agilent 5975 MSD. **Figure 8** below shows an image of the GCMS used in this experiment.

Figure 8: An image of the Agilent GC and MSD system models.

The succinic acid was chemically converted to DMS using the method described at Section 1.1 above. DMS in pH 4 and 10 standards were prepared using a 99% pure DMS from Acros Organics. These standards were prepared at a concentration of approximately 1g/L. The pH standards were extracted into ethyl acetate layer. The liquid-liquid extraction was conducted by having 5mL of the pH standard with 5mL of ethyl acetate. This solution was vortexed for 30s and from the 5mL ethyl acetate layer (top layer), 1mL of it was obtained and dispensed into 2mL vials. The column that was used to analyze everything through the GCMS was an Agilent J&W GC Columns HP-5MS capillary column with a length of 30m, diameter of 0.250mm, and a film size of 0.25µm. The carrier gas for the GCMS was helium.

Table 2: Oven parameters for pH standard analysis. The post run was for 1min at 280°C.

Rate (°C/min)	**Temperature (°C)**	**Hold Time (min)**
-	50	5.35
5.3	120	0
50	270	5

The GCMS method used to analyze the pH standards had the oven setup to start at 50°C and hold that temperature for 5.35min. Then, at a ramp rate of 5.3°C/min. the temperature was increased to 120°C. The next ramp rate was programmed to 50°C/min to 270°C and held for 5min. The oven method ended with a post run of 1min at 280°C to bake everything of the column. The total run time for the method was 27.558min. The injection size for each sample was 1µL. The inlet temperature was set at 250°C. The split ratio is 10:1 with the split flow being 15mL/min. The MSD temperature was set at a constant temperature of 280°C. The column had a constant flow rate of 1.5mL/min and a solvent delay of 4.50min.

After the esterification, both the gas phase and liquid phase of the reaction were analyzed through the GCMS. The gas phase was obtained by penetrating the crimp vial septa using an air-tight syringe and obtaining around 1mL of sample. This gas phase sample was then manually injected into the GC through the inlet. An image of the air-tight syringe can be observed in **Figure 9** below.

Figure 9: An image of the air-tight syringe used for manual injection into the GCMS.

The chemical workup after the esterification reaction for the liquid phase was performed by obtaining 5mL of the reacted mixture and added into another crimp vial, followed by the addition of sodium bicarbonate to neutralize the solution. Then 5mL of ethyl acetate solution was added into the vial. This mixture was vortexed for 40s. From the vortexed mixture, 1mL of it was

extracted into a 1.5mL Eppendorf centrifuge tube and centrifuged at 13,000/min for 6min. From the centrifuge tube, 500µL was dispensed into a 2mL vial and ran on the GCMS through ALS.

Table 3: Oven parameters for the analysis of liquid and gas phases of the succinic acid esterification. The post run was for 1min at 280°C.

Rate (°C/min)	**Temperature (°C)**	**Hold Time (min)**
-	85	0
70	150	0
70	220	3

The esterification reaction standards, both gas and liquid phases, were analyzed through the same method, except one was through ALS and the other through manual start. The oven temperature started at 85°C. At 70°C/min, the temperature increased to 150°C. Then, at a ramp rate of 70°C/min, the temperature was increased to 220°C and held for 3min. The post run temperature was at 280°C for 1min to bake the column. The total run time for the method is 5.9286min. The injection size for each sample was 1µL. The inlet temperature was set at 250°C. The MSD temperature was set at 280°C. The split ratio was 10:1 and the split flow was 15mL/min. The flow rate in the column was set at 1.5mL/min.

Section 1.4: HSGC Parameters and Sample Preparation

The sample preparation for the HSGC was done exactly as mentioned in Section 1.1 above. The samples were ran using a Shimadzu GC-2010 with a Shimadzu HS-20 system. The type of column used in the HSGC is the exact same brand that was used in the GCMS. The brand of the column can be found in Section 1.3. An image of the instrument can be observed in **Figure 10** below.

Table 4: HS20 oven parameters.

Parameters	**Values**
Mode:	Loop
Oven Temp.:	95°C
Sample Line Temp.:	225°C
Transfer Line Temp.:	250°C
Shaking Level:	2
Multi Injection Count:	1
Pressurize Gas Pressure:	240kPa
Equilibrating Time	0.50min
Pressurizing Time	0.10min
Pressure Equilib. Time:	0.20min
Load Time:	0.50min
Load Equilib. Time:	0.10min
Injection Time:	0.35min
Needle Flush Time:	0.10min
Gc Cycle Time:	3.50min

Table 5: HS20 flow parameters.

Parameters	**Values**
Injection Mode:	Split
Flow Control Mode:	Pressure
Pressure:	135kPa
Total Flow:	16.5mL/min
Column Flow:	1.50mL/min
Linear Velocity:	36.5cm/sec
Purge Flow:	0
Split Ratio:	10

Table 6: GC oven parameters.

Rate (°C/min)	**Temperature (°C)**	**Hold Time (min)**
-	100	0
60	150	0
60	250	0

Table 7: FID flow and temperature parameters. The carrier gas is helium.

Parameters	**Values**
Temperature:	260°C
Sampling Rate:	40msec
Stop Time:	2.50min
H_2 Flow:	40mL/min
Makeup Flow:	30mL/min
Air Flow:	400mL/min

Figure 10: An image showing the Shimazu HSGC used for the experiment.

The tables above show the HSGC run parameters. The oven parameters for the HS section were that the vial was to be heated at 95°C for 0.50min (this parameter is called equilibrating time on the HSGC). During this time the sample was also shaken at level 2. The pressurizing time and pressure equilibration time were 0.10 and 0.20min, respectively. The pressurize gas pressure was set at 240kPa. The load time and load equilibration time were set at 0.50 and 0.10min, respectively. Injection time was set at 0.35min with multi-injection count at one. The sample line and transfer line temperatures were set at 225.0 and 250°C, respectively. The post run (also called the GC cycle time) was set at 3.50min, which was 1min more than the run time. The split ratio was chosen to be

10:1. The flow control mode was chosen to be pressure and the column flow was set at 1.50mL/min at 135.0kPa.

The GC oven was set to start at 100°C. At 60°C/min, the temperature ramped up to 150°C. Then, at 60°C/min, the temperature was ramped up to 250°C. The equilibration time was set up to 1min. The run time was 2.50min. The total run time, including the times from the HS parameters, added up to 5.25min. The FID temperature was set at 260°C. The carrier gas was helium. The hydrogen flow was set at 40mL/min. The make-up flow was set at 30mL/min. The air flow was set at 400mL/min. From the HS section, and then to details, the check system ready box was unchecked. The reason for this was that the sample and transfer line temperatures sometimes altered randomly in the decimal region by 1-2. This kept changing the total run time because the next sample did not run until these two temperatures were exactly at the values they were set at.

Section 1.5: CIMS Parameters and Sample Preparation

The sample preparation for the CIMS was performed exactly as mentioned in Section 1.1 above. The chemical workup after the esterification is performed the same way as mentioned in Section 1.3 for the GCMS, however, the solvent used for liquid-liquid extraction here was cyclohexane, instead of ethyl acetate. Nitrogen was the carrier gas and benzene were the reagent gas. The CIMS was placed in positive ion mode. A 10µL GC needle was used to inject around ¼ of a microliter manually. The ion molecular reactor (IMR) was at a pressure of 45torr. The benzene had a flow of 0.51LPM air. **Figure 11** below contains the image of the CIMS instrument used.

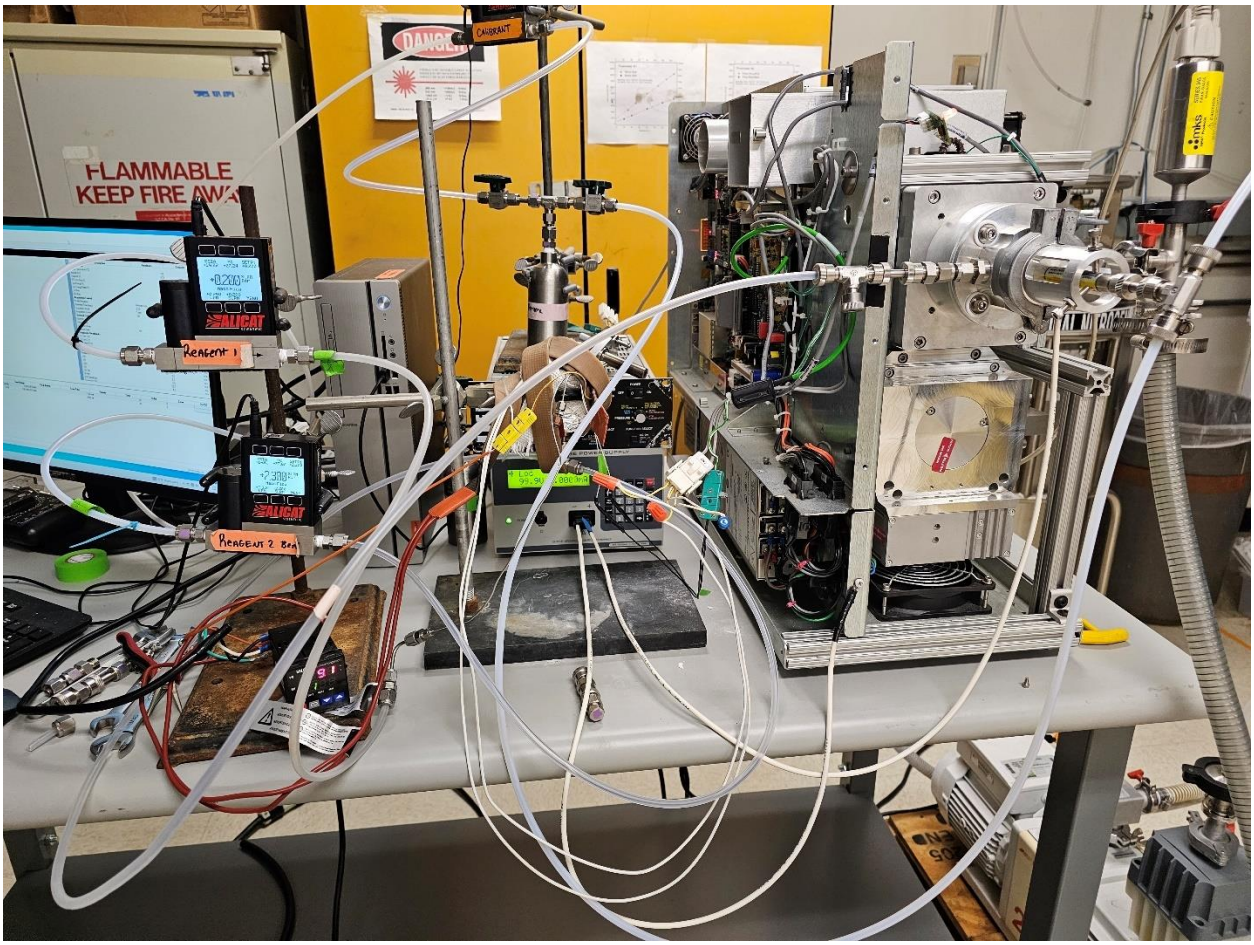
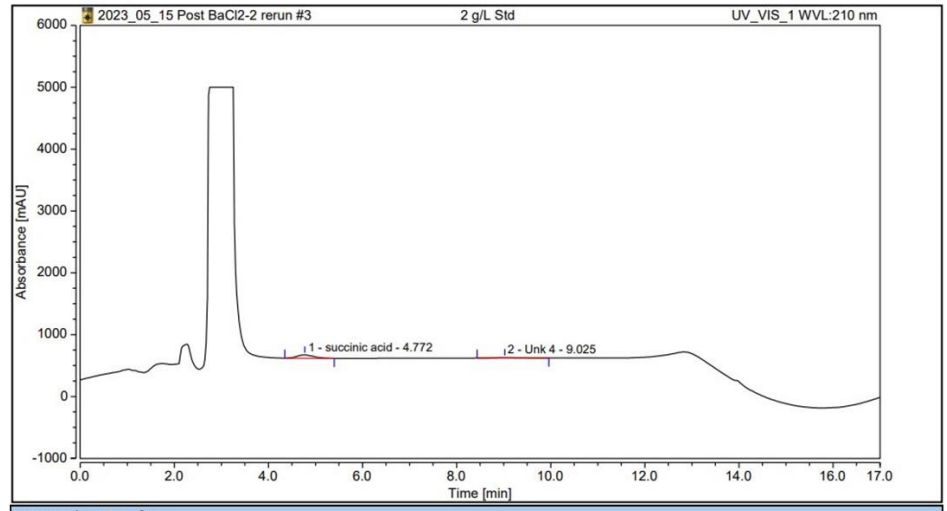


Figure 11: An image of the CIMS instrument used for this experiment.

Chapter 2 RESULTS

Section 2.1: HPLC Results for the SA

Figure 12 below shows the chromatogram acquired from the HPLC for the 2g/L SA standard in BG-11 medium. The run method that was used for this experiment has been done before, which is why the method contains pre-coded locations for peaks of interest. It was discovered before that the succinic acid peak usually had a retention time around 4.7min. The other peaks that the method integrates were never analyzed further because they were outside the scope of the research, hence the name unknown. However, they were still integrated to show they exist in the chromatography run. The large peak, that has a retention time around 3min, was noticed to appear in standards whenever formic acid was added to them.



Integration Results

No.	Peak Name	Retention Time min	Area mAU*min	Height mAU	Relative Area %	Relative Height %	Amount g/L
1	succinic acid	4.772	22.279	53.854	80.58	86.48	n.a.
n.a.	Unk 1	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Unk 2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
n.a.	Unk 3	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
2	Unk 4	9.025	5.368	8.423	19.42	13.52	n.a.
Total:			**27.647**	**62.277**	**100.00**	**100.00**	

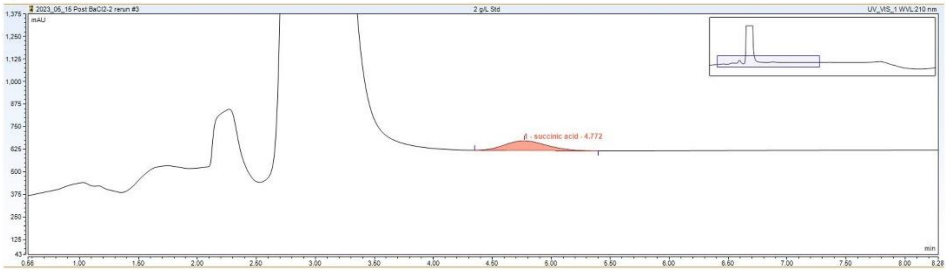


Figure 12: The first image is HPLC chromatogram for the 2g/L SA standard and the chromatogram's integration table. The second image is the zoomed in version of the chromatogram to the succinic acid peak.

Section 2.2: GCMS Results for pH Standards

In **Figure 13**, the NIST library states that the peak in 11.6min is the DMS peak and the peak in 19.3min is a phthalic acid. The pH 4 and pH 10 standards had a color indicator added to them. Phthalic acid is usually used for dyes, which is probably what is also in the color indicator. In **Figure 16**, it can be observed that the DMS in pH 10 solution has a drastic decrease in peak height between a one-day period.

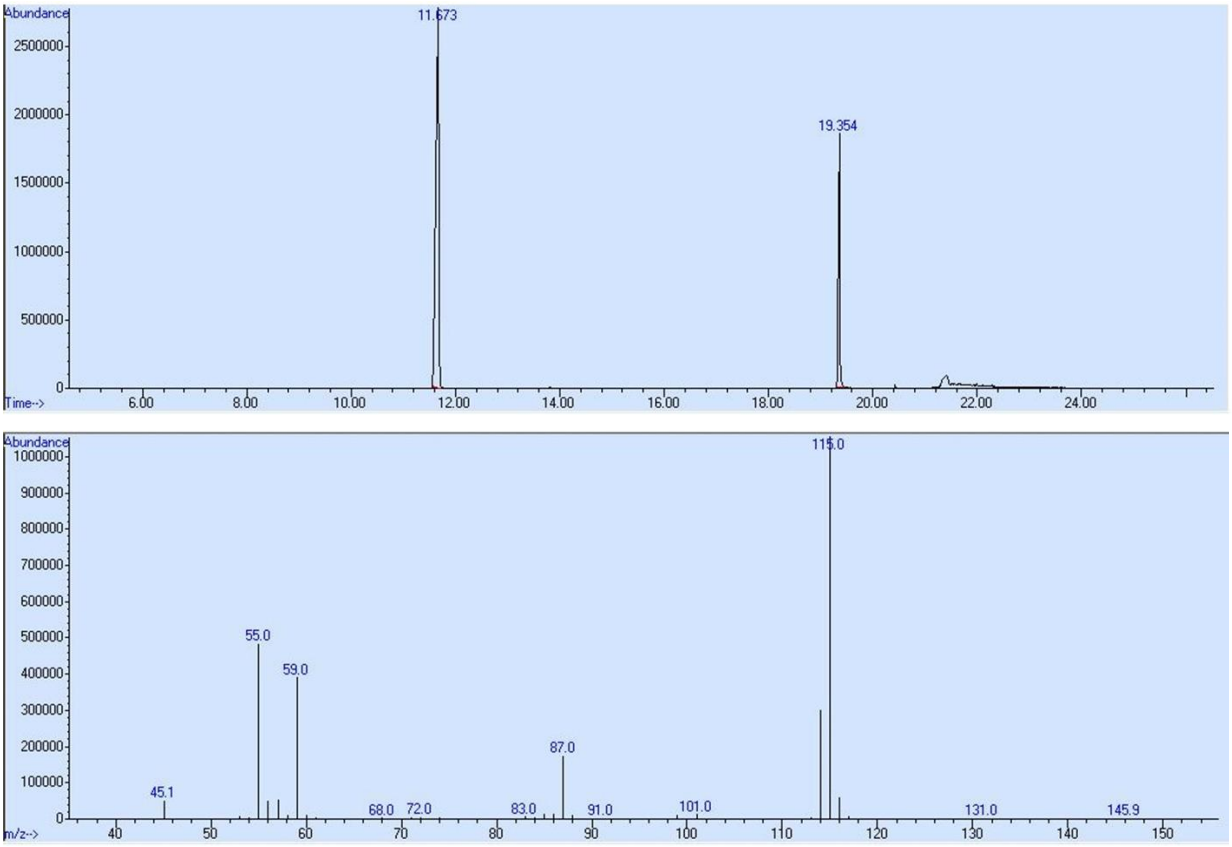


Figure 13: The first image is the GCMS chromatogram result for the DMS in pH 4 standard. The second image is the MS data for the first peak at 11.6min. The third image (at the bottom) is the NIST library search for the peak at 11.6min. The fourth image (at the bottom) is the NIST library search for the peak at 19.3min.

Figure 13 Continued.

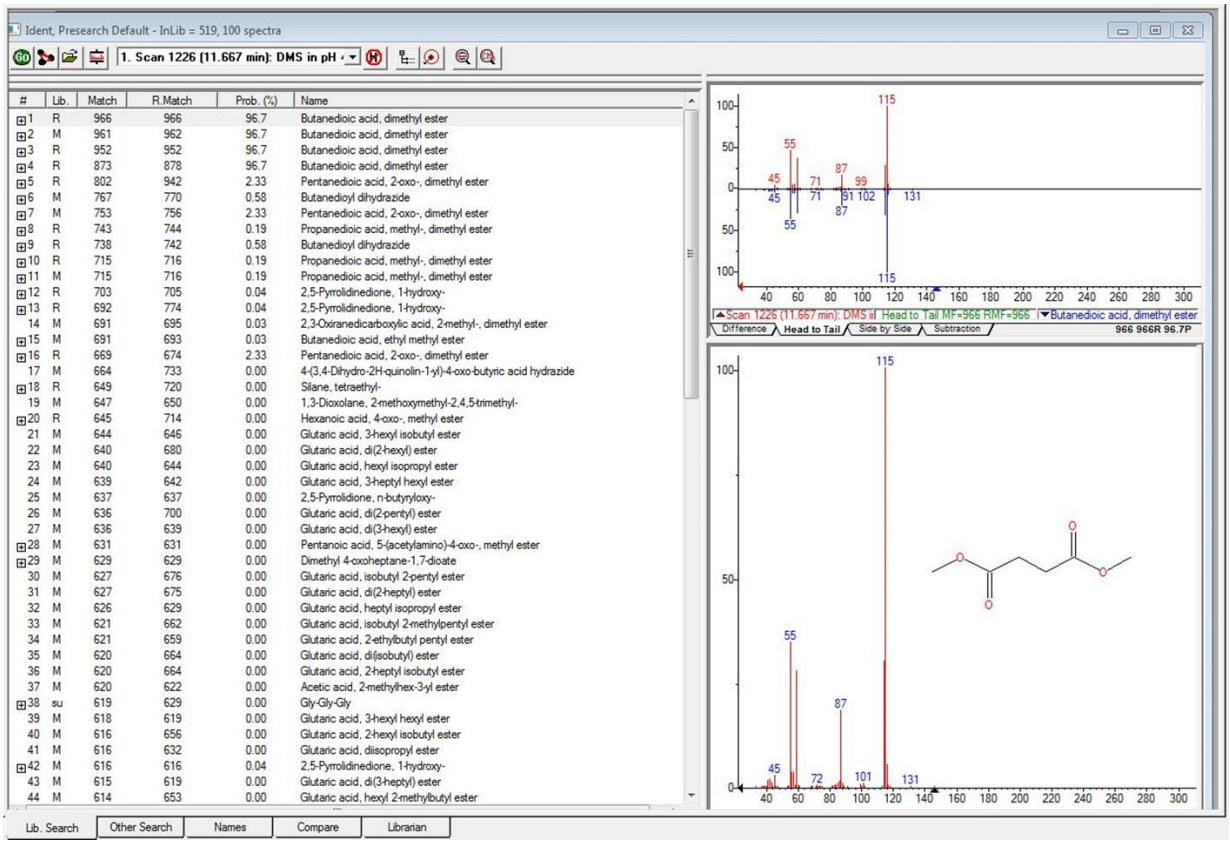
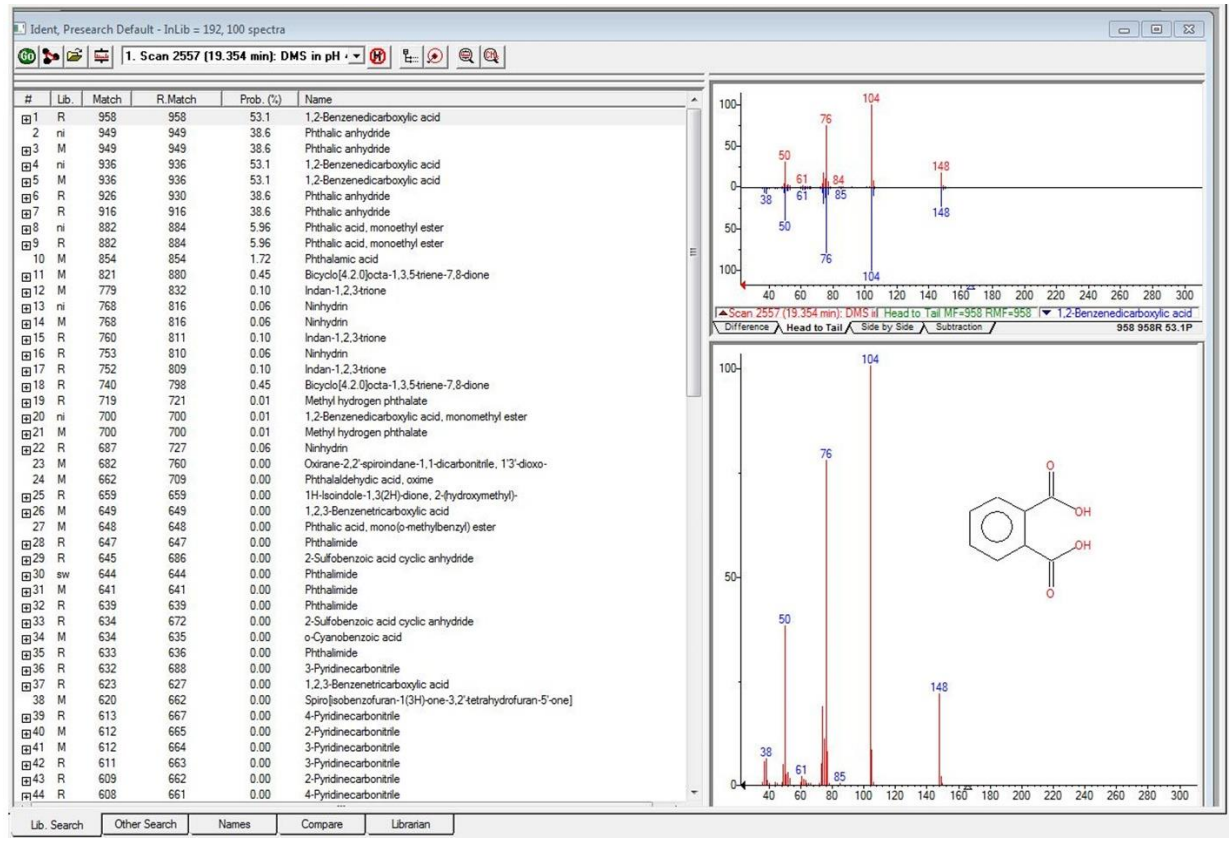


Figure 13 Continued.



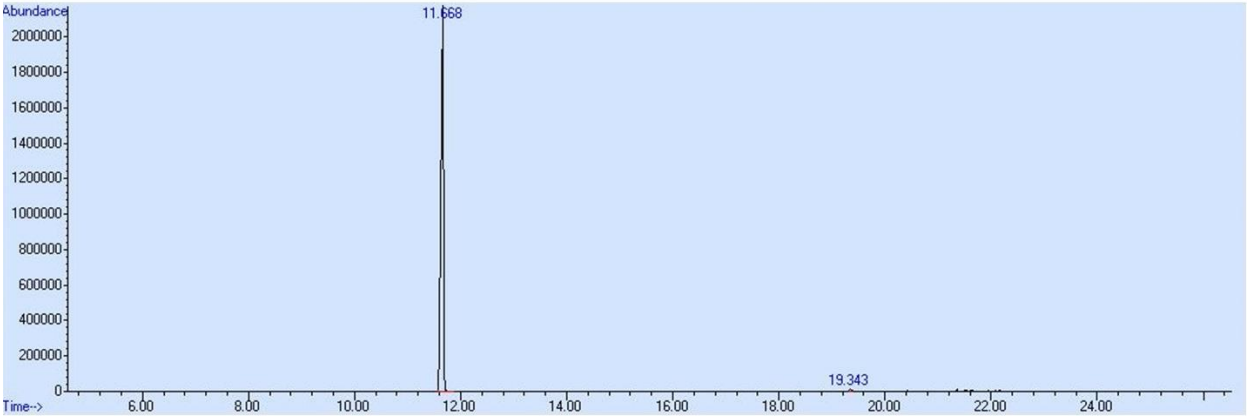
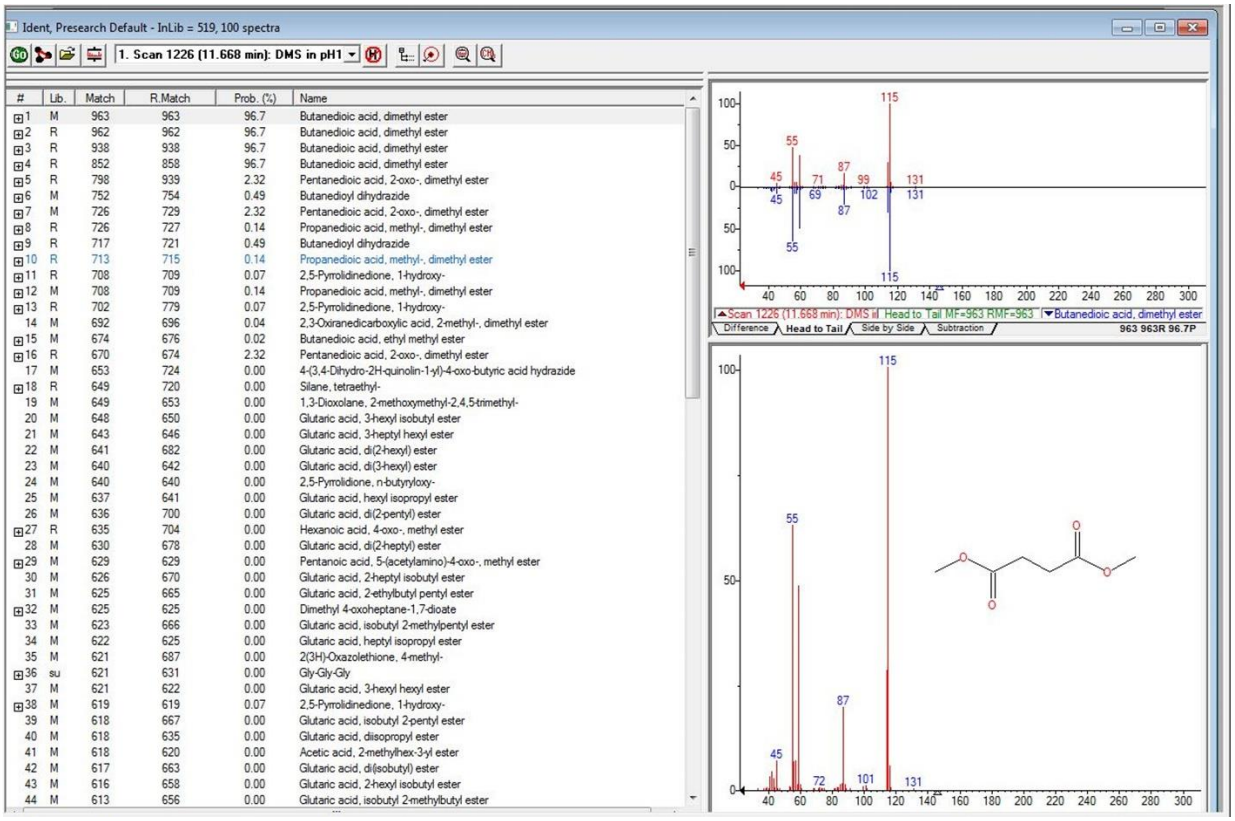


Figure 14: The first image is the GCMS chromatogram of the DMS in pH 10 standard. The second image (at the bottom) is the NIST library search of the peak at 11.6min.

Figure 14 Continued.



#	Lib.	Match	R.Match	Prob. (%)	Name
1	M	963	963	96.7	Butanedioic acid, dimethyl ester
2	R	962	962	96.7	Butanedioic acid, dimethyl ester
3	R	938	938	96.7	Butanedioic acid, dimethyl ester
4	R	852	858	96.7	Butanedioic acid, dimethyl ester
5	R	798	939	2.32	Pentanedioic acid, 2-oxo-, dimethyl ester
6	M	752	754	0.49	Butanedioyl dihydrazide
7	M	726	729	2.32	Pentanedioic acid, 2-oxo-, dimethyl ester
8	R	726	727	0.14	Propanedioic acid, methyl-, dimethyl ester
9	R	717	721	0.49	Butanedioyl dihydrazide
10	R	713	715	0.14	Propanedioic acid, methyl-, dimethyl ester
11	R	708	709	0.07	2,5-Pyrrolidinedione, 1-hydroxy-
12	M	708	709	0.14	Propanedioic acid, methyl-, dimethyl ester
13	R	702	779	0.07	2,5-Pyrrolidinedione, 1-hydroxy-
14	M	692	696	0.04	2,3-Oxiranedicarboxylic acid, 2-methyl-, dimethyl ester
15	M	674	676	0.02	Butanedioic acid, ethyl methyl ester
16	R	670	674	2.32	Pentanedioic acid, 2-oxo-, dimethyl ester
17	M	653	724	0.00	4-(3,4-Dihydro-2H-quinolin-1-yl)-4-oxo-butyric acid hydrazide
18	R	649	720	0.00	Silane, tetraethyl-
19	M	649	653	0.00	1,3-Dioxolane, 2-methoxymethyl-2,4,5-trimethyl-
20	M	648	650	0.00	Glutaric acid, 3-hexyl isobutyl ester
21	M	643	646	0.00	Glutaric acid, 3-heptyl hexyl ester
22	M	641	682	0.00	Glutaric acid, di(2-hexyl) ester
23	M	640	642	0.00	Glutaric acid, di(3-hexyl) ester
24	M	640	640	0.00	2,5-Pyrrolidione, n-butyryloxy-
25	M	637	641	0.00	Glutaric acid, hexyl isopropyl ester
26	M	636	700	0.00	Glutaric acid, di(2-pentyl) ester
27	R	635	704	0.00	Hexanoic acid, 4-oxo-, methyl ester
28	M	630	678	0.00	Glutaric acid, di(2-heptyl) ester
29	M	629	629	0.00	Pentanoic acid, 5-(acetylamino)-4-oxo-, methyl ester
30	M	626	670	0.00	Glutaric acid, 2-heptyl isobutyl ester
31	M	625	665	0.00	Glutaric acid, 2-ethylbutyl pentyl ester
32	M	625	625	0.00	Dimethyl 4-oxoheptane-1,7-dioate
33	M	623	666	0.00	Glutaric acid, isobutyl 2-methylpentyl ester
34	M	622	625	0.00	Glutaric acid, heptyl isopropyl ester
35	M	621	687	0.00	2(3H)-Oxazolethione, 4-methyl-
36	su	621	631	0.00	Gly-Gly-Gly
37	M	621	622	0.00	Glutaric acid, 3-hexyl hexyl ester
38	M	619	619	0.07	2,5-Pyrrolidinedione, 1-hydroxy-
39	M	618	667	0.00	Glutaric acid, isobutyl 2-pentyl ester
40	M	618	635	0.00	Glutaric acid, diisopropyl ester
41	M	618	620	0.00	Acetic acid, 2-methylhex-3-yl ester
42	M	617	663	0.00	Glutaric acid, di(isobutyl) ester
43	M	616	658	0.00	Glutaric acid, 2-hexyl isobutyl ester
44	M	613	656	0.00	Glutaric acid, isobutyl 2-methylbutyl ester

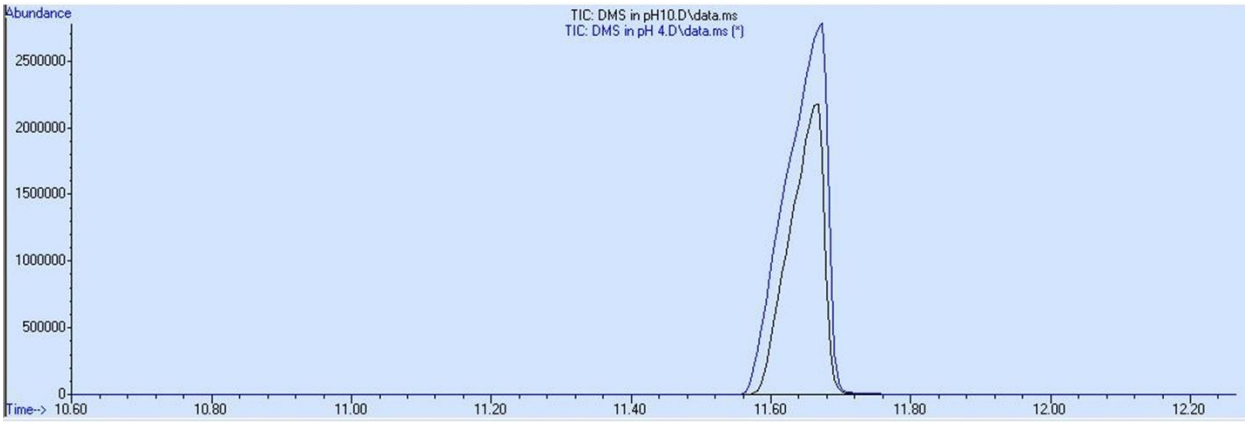


Figure 15: The image displays the chromatography overlay between the DMS in pH 4 and pH 10 standards.

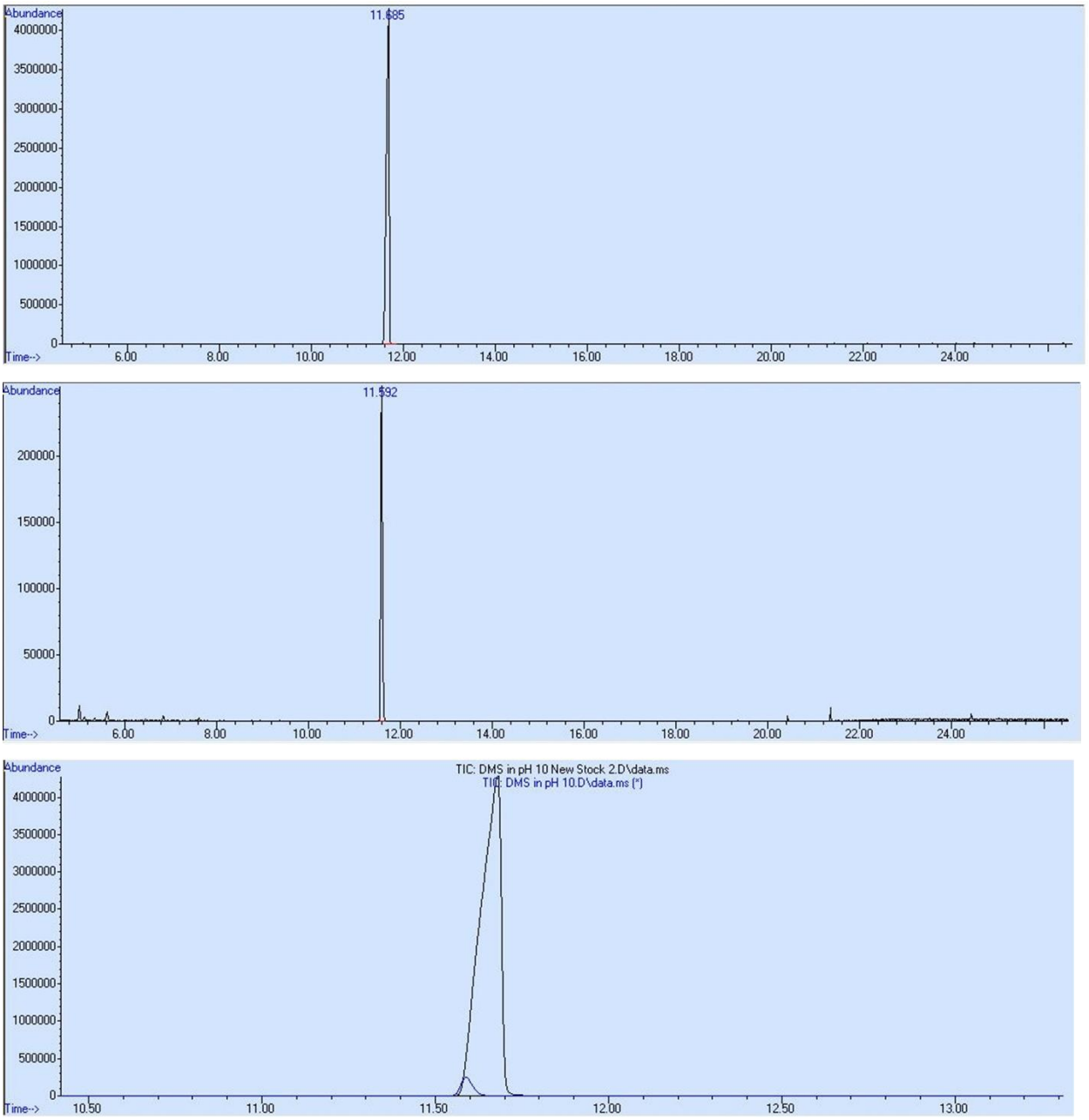


Figure 16: The first image is GCMS chromatogram for the new stock of DMS in pH 10 standard on September 8th. The second image is the GCMS chromatogram of the same stock on the next day, September 9th. The third image is the overlay of the two chromatograms showing the DMS peaks.

Section 2.3: GCMS Results of the SA Esterification for the Liquid and Gas Phases

The NIST library for both the liquid phase and gas phase for the succinic acid standard showed that the esterification in the crimp vial worked. This is indicated by the DMS peak at 1.5 and 1.6min.

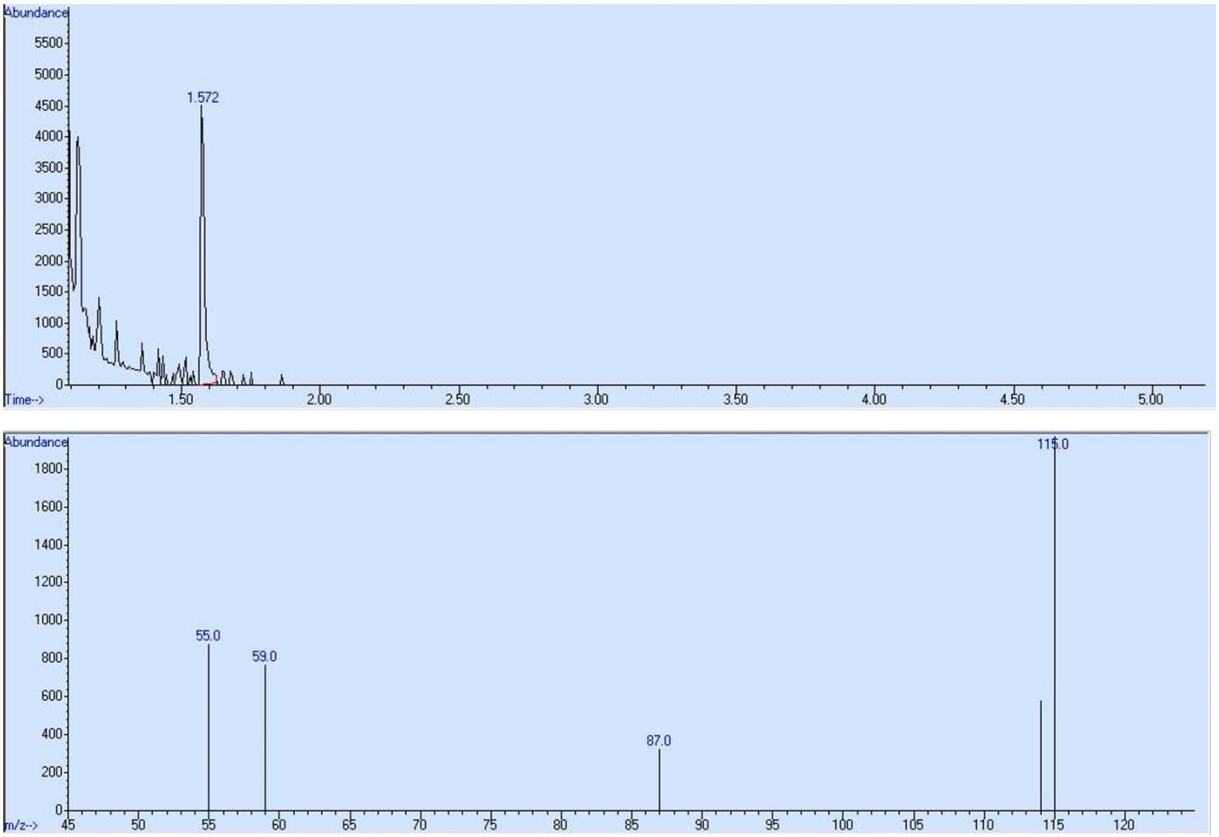
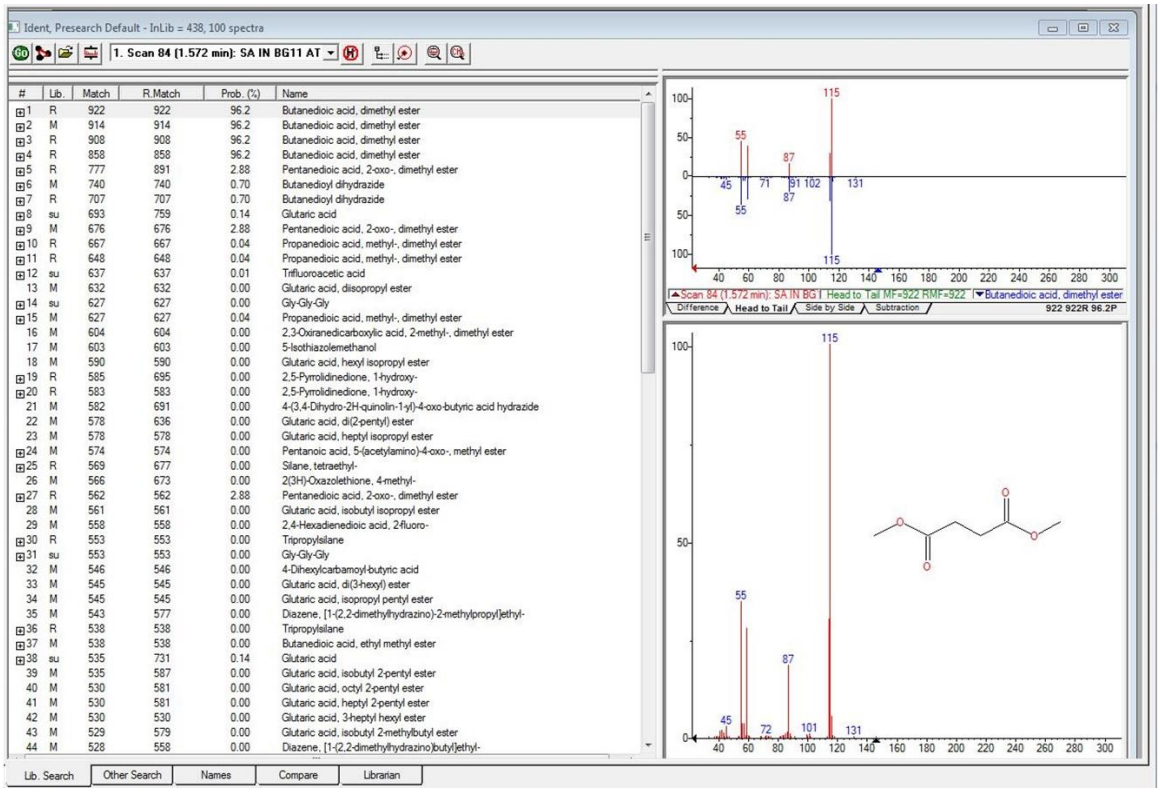


Figure 17: The first image shows the GCMS chromatogram of the manually injected 20g/L esterified SA's gas phase. The second image shows the MS data of the peak at 1.5min. The third image (at the bottom) shows the NIST library search of the peak at 1.5min.

Figure 17 Continued.



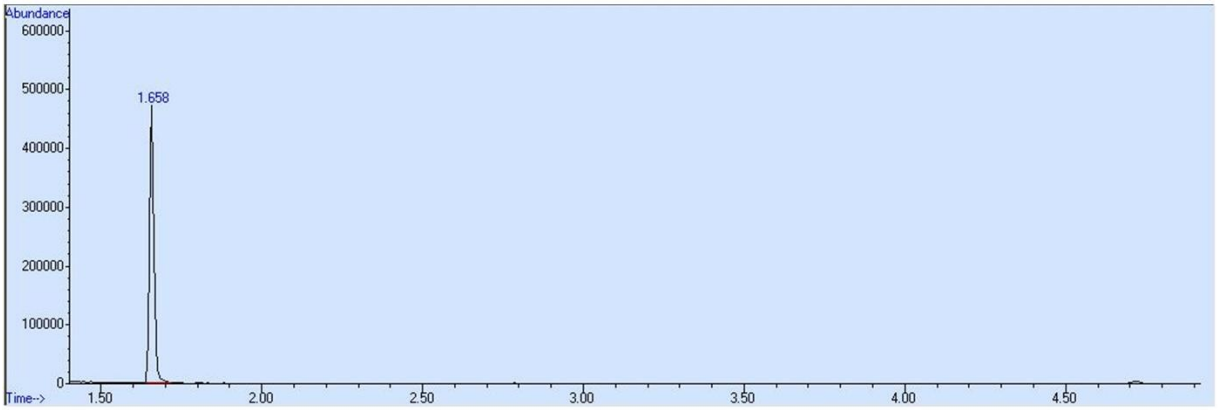
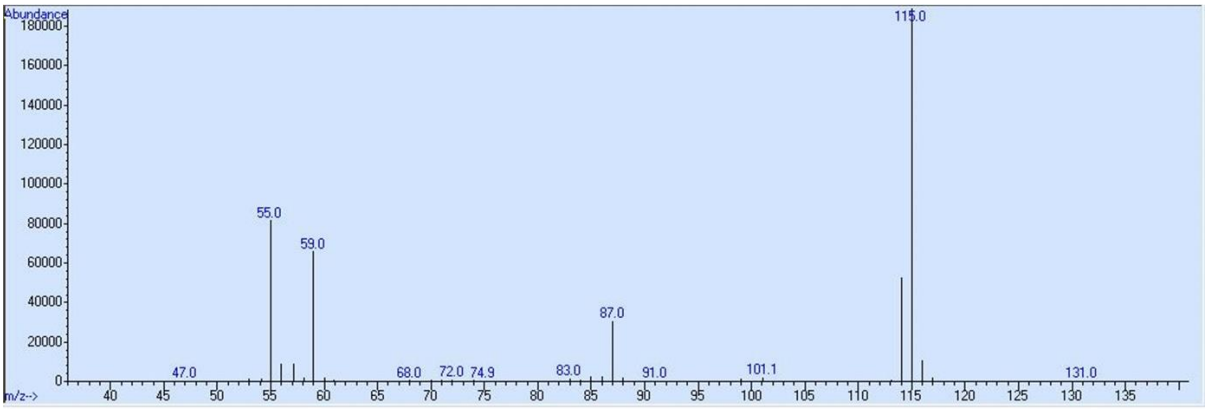
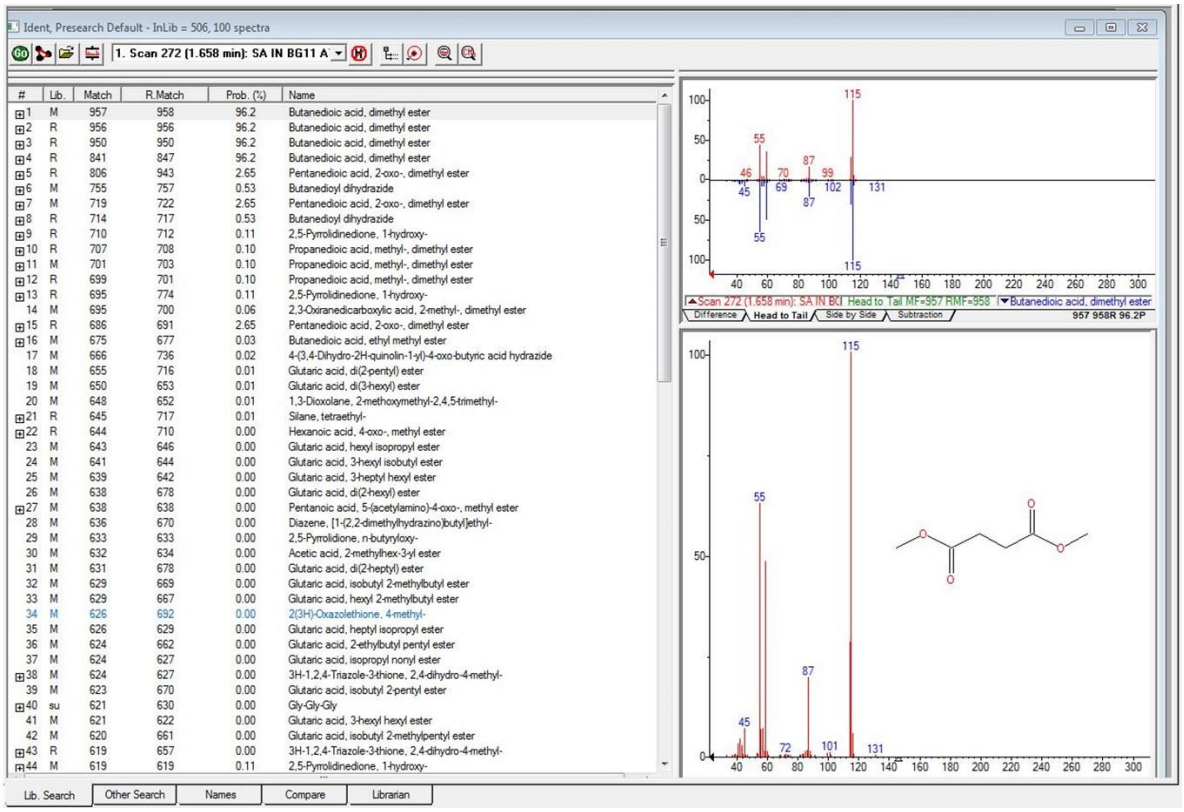


Figure 18: The first image shows the GCMS chromatogram of the 20g/L esterified SA's liquid phase. The second image (at the bottom) shows the MS data of the peak at 1.6min. The third image (at the bottom) shows the NIST library search for the peak at 1.6min.

Figure 18 Continued.





Section 2.4: HSGC Results

In **Figure 19**, the overlay of esterified SA standard chromatograms can be observed. The SA standard concentrations of 1, 5, 10, 15, and 20g/L match up with the black, pink, blue, brown, and green chromatograms, respectively.

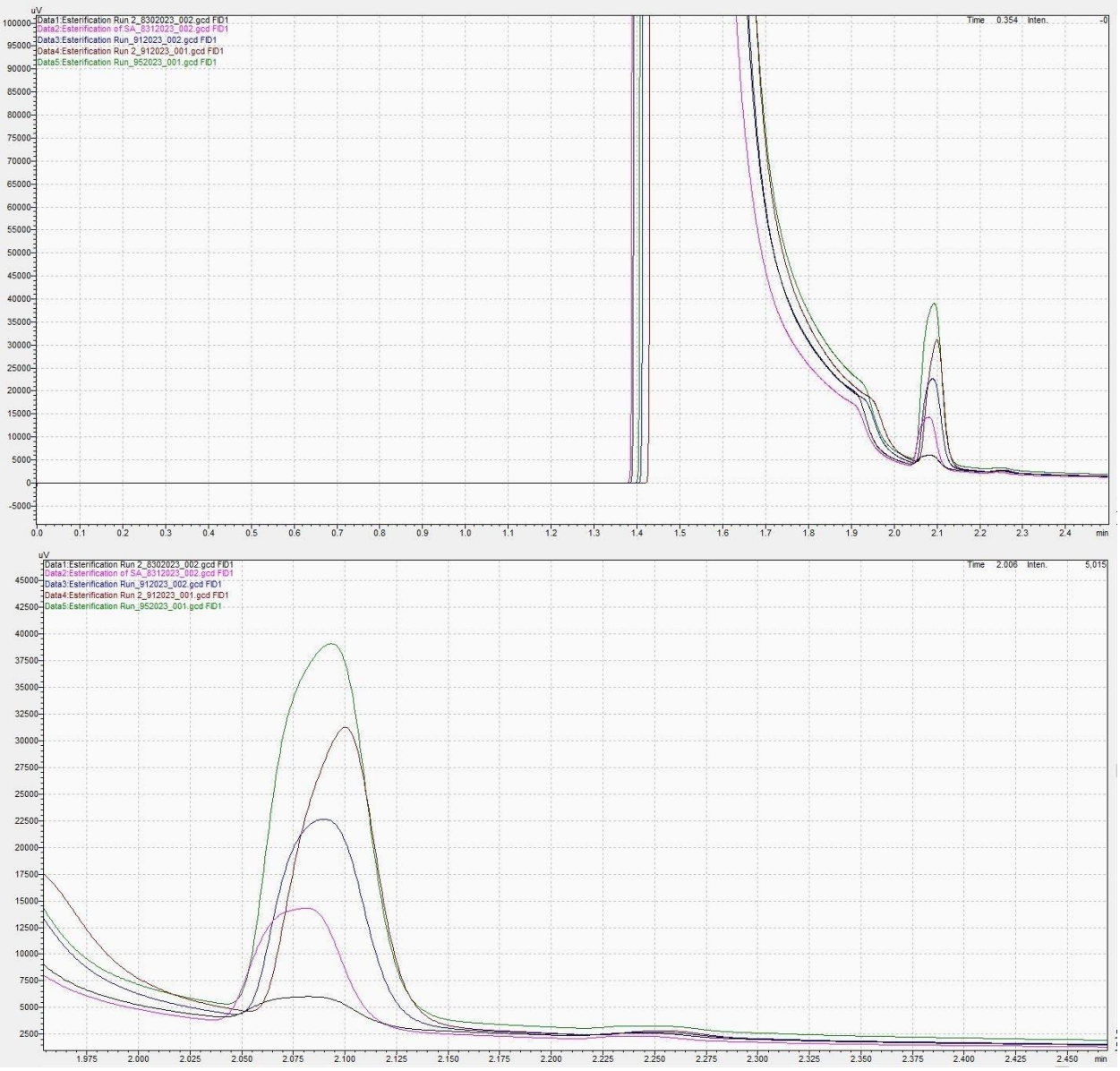


Figure 19: The first image above is the overlay of 1, 5, 10, 15, and 20g/L esterified SA standards. The second image below is the zoomed in version to the DMS peaks.

In **Figure 20**, the first pink peak, for the 99% pure DMS, near the solvent peak is from the carryover of methanol peaks from the methanol, positive control, and negative control. This is because the 99% pure DMS sample ran last. The second blue peak for the positive control matches up with the second peak from the 99% pure DMS. This would mean that specific peak is the DMS peak.

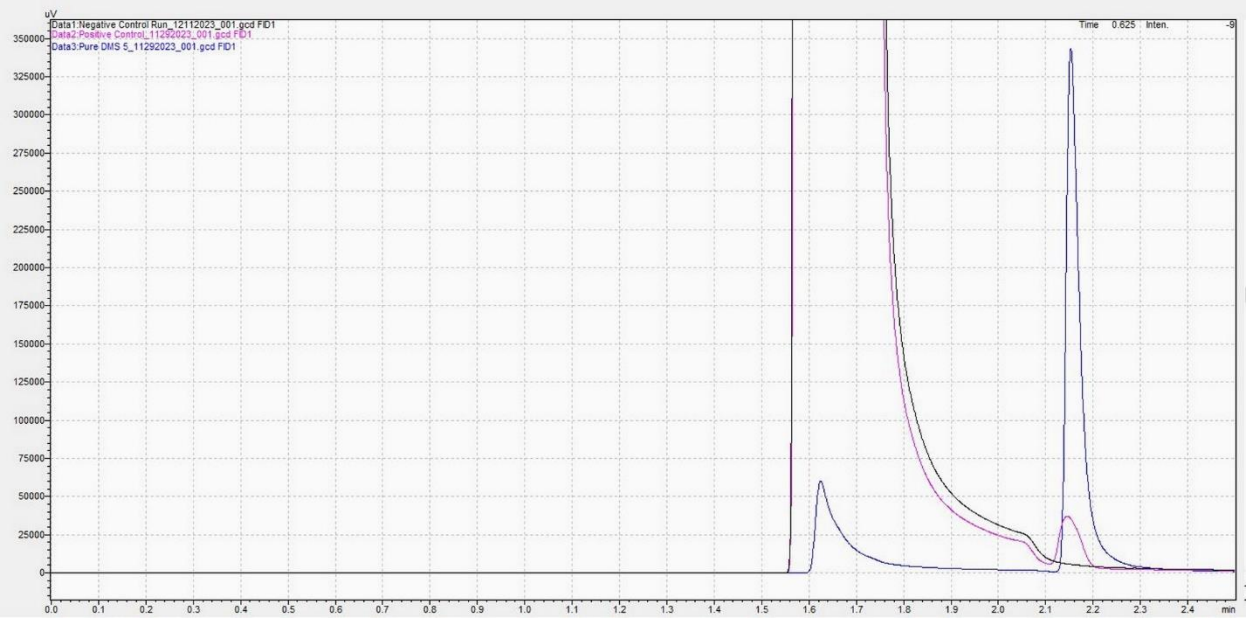


Figure 20: The image above shows the overlay HSGC chromatograms of 99% pure DMS (blue), positive control (pink), negative control (black).

Section 2.5: HSGC Results and calibration Curve of the Esterification Standards

The graph in **Figure 21** shows the peak areas of the DMS peak around 2.1min. The R^2 of the graph is 0.9764, meaning the data fits the linear model well. The concentrations used to make the external calibration were 1, 5, 10, 15, and 20g/L. However, the concentrations plotted in the graph are the actual concentrations of the standards. **Table 8** below shows the actual concentrations of the standards. **Table 9** below shows the areas of each individual trial of esterified SA standards

with their respective average areas. The average areas for each standard were used to plot the calibration curve in **Figure 21**.

Table 8: Theoretical concentrations against the actual concentrations used for the experiment.

Theoretical Concentration (g/L)	**Actual Concentration (g/L)**
1	1.129
5	4.97
10	10.09
15	14.99
20	19.66

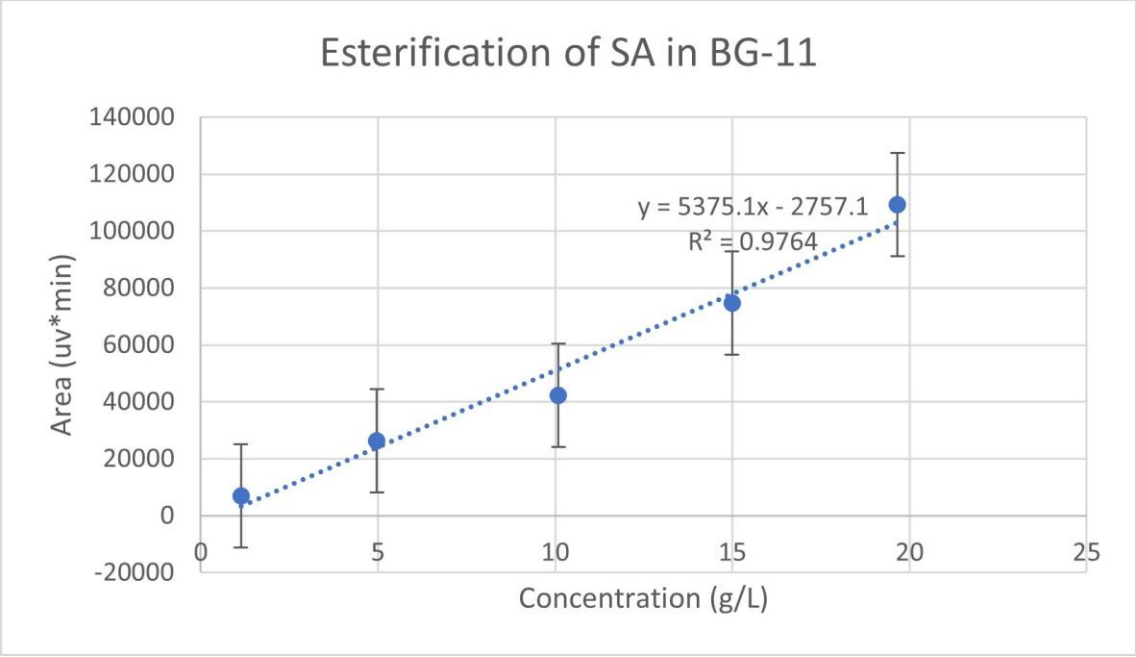


Figure 21: The graph above is the calibration curve for the esterified SA standards with error bars.

Table 9: Areas for each standard run on the HSGC and their respective averages.

Concentrations (g/L)	**Areas (µv*min)**
1.129g/L:	5245
	6981
	8868
Average:	7031.3
4.966g/L:	18887
	30097
	29835
Average:	26273
10.09g/L:	24219
	51567
	50956
Average:	42247.3
14.99g/L:	68053
	99797
	55932
Average:	74594
19.66g/L:	109381
	101313

	117150
Average:	109281.3

Table 9 continued.

Section 2.6: CIMS Result of Cyclohexane

In **Figure 22**, the spectra of the cyclohexane injection can be observed. The peak around 78m/z is the reagent gas, benzene. The peaks at approximately 81m/z and 92m/z are from carry over. The zoomed in spectra of the cyclohexane to the 140 to 150m/z is to show what the peak around 146m/z, the DMS peak, looks like.

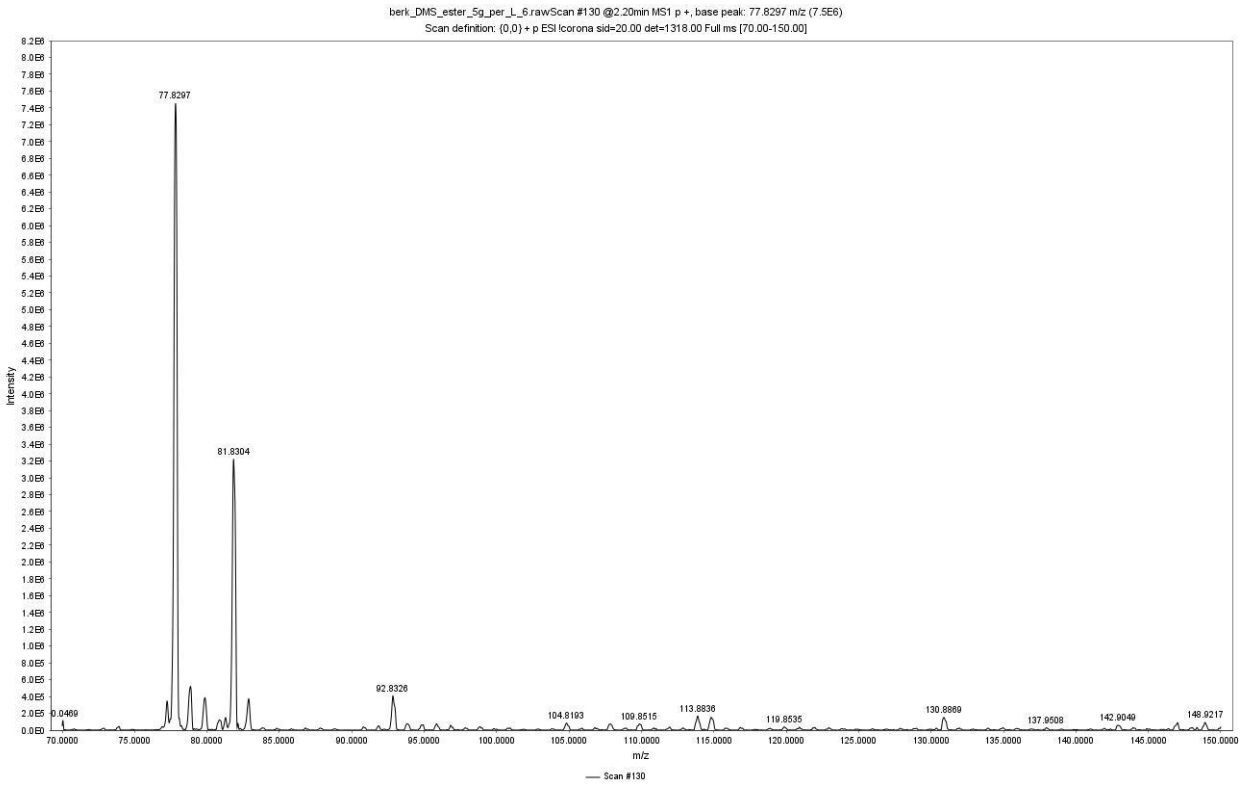
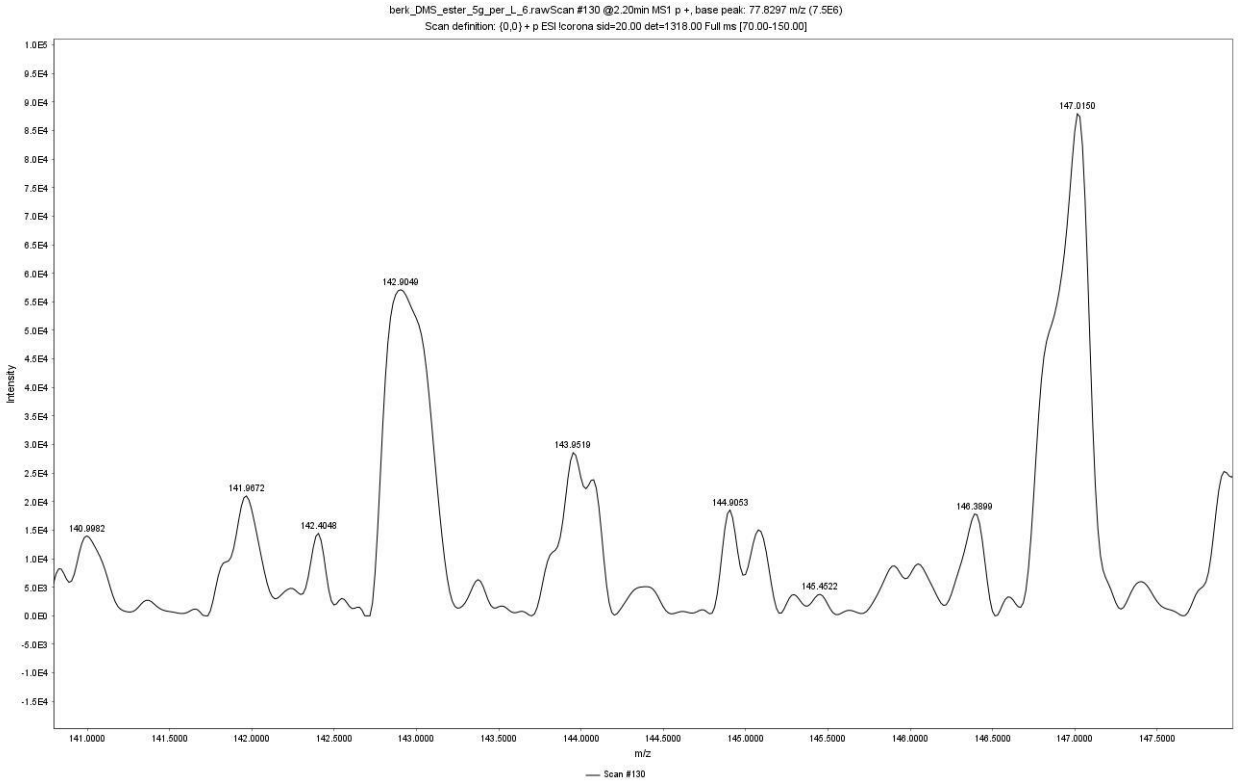


Figure 22: The first image above shows the full spectra of injecting cyclohexane from 70 to 150m/z. The second image (below) is the magnification of the first image to 140 to 150m/z.

Figure 22 continued.



Section 2.7: CIMS Results for Positive Control

The images in **Figure 23** show the CIMS spectra between 140 to 150m/z before and after injecting the 20g/L of DMS in cyclohexane positive control. The peak intensity is around 3.6E4 for 146.9m/z before the positive control injection. After the positive control is injected, the peak intensity is around 1.4E7 for 146.8m/z.

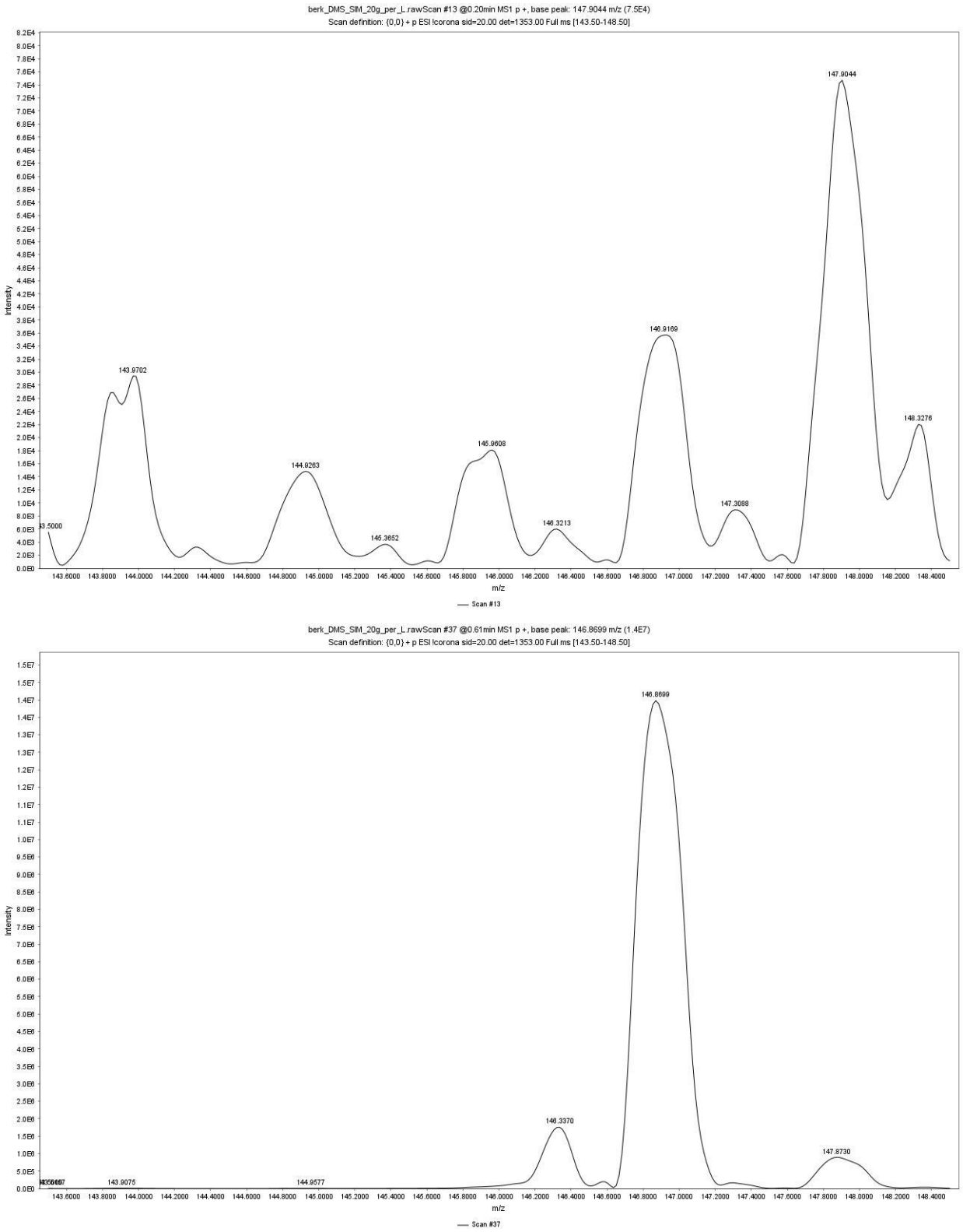


Figure 23: The first image is the spectra from 140 to 150m/z before injecting the positive control and the second image is the spectra after injecting the positive control.

Section 2.8: CIMS Results for 5, 10, 15, and 20g/L Esterification Standards

In **Figure 24** and **Figure 25**, the time plot and spectra for 5g/L esterification standard can be observed, respectively. The spectra shown in **Figure 25** is from the peak at 2.06min from the time plot. From the spectra, the DMS peak is shown around 146.9m/z at 2.4E6 intensity. The DMS also went through fragmentation in the CIMS, resulting in a peak around 114.8m/z at 4.0E6 intensity.

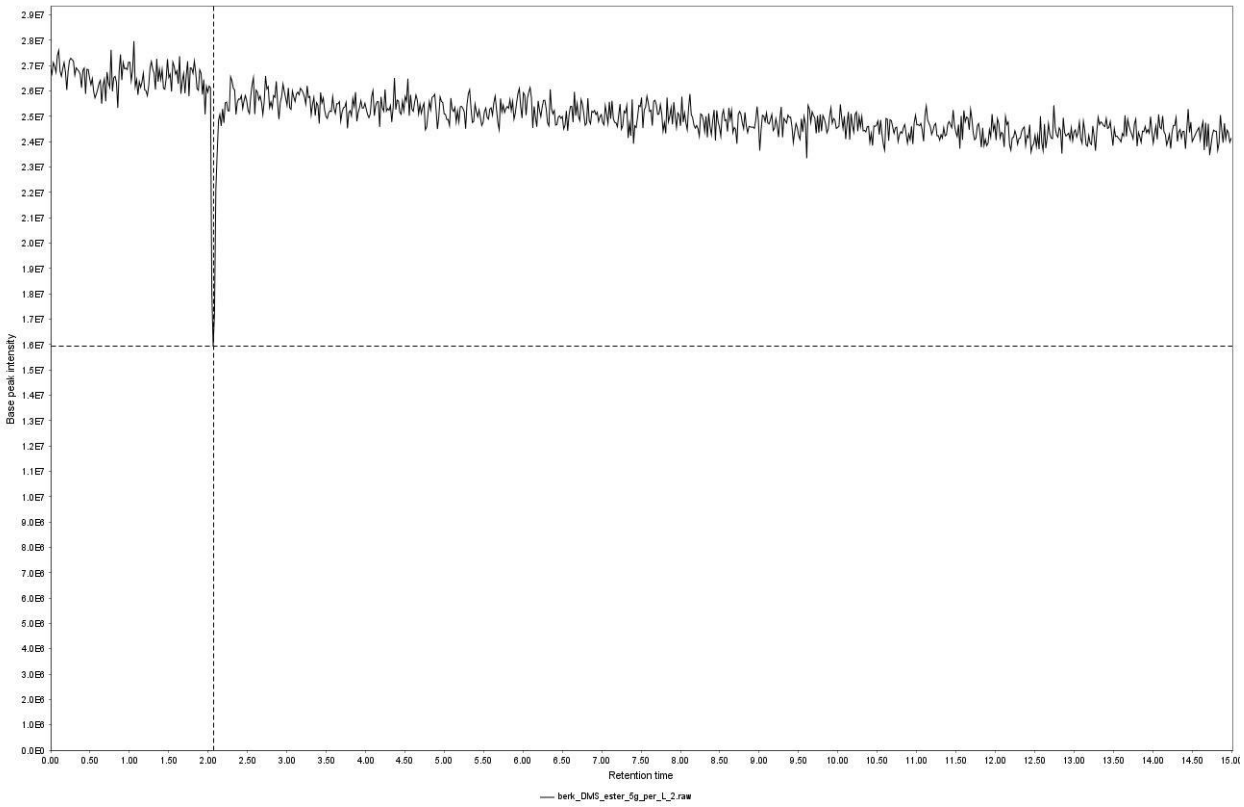


Figure 24: Time plot of 5g/L SA in BG-11 esterification standard.

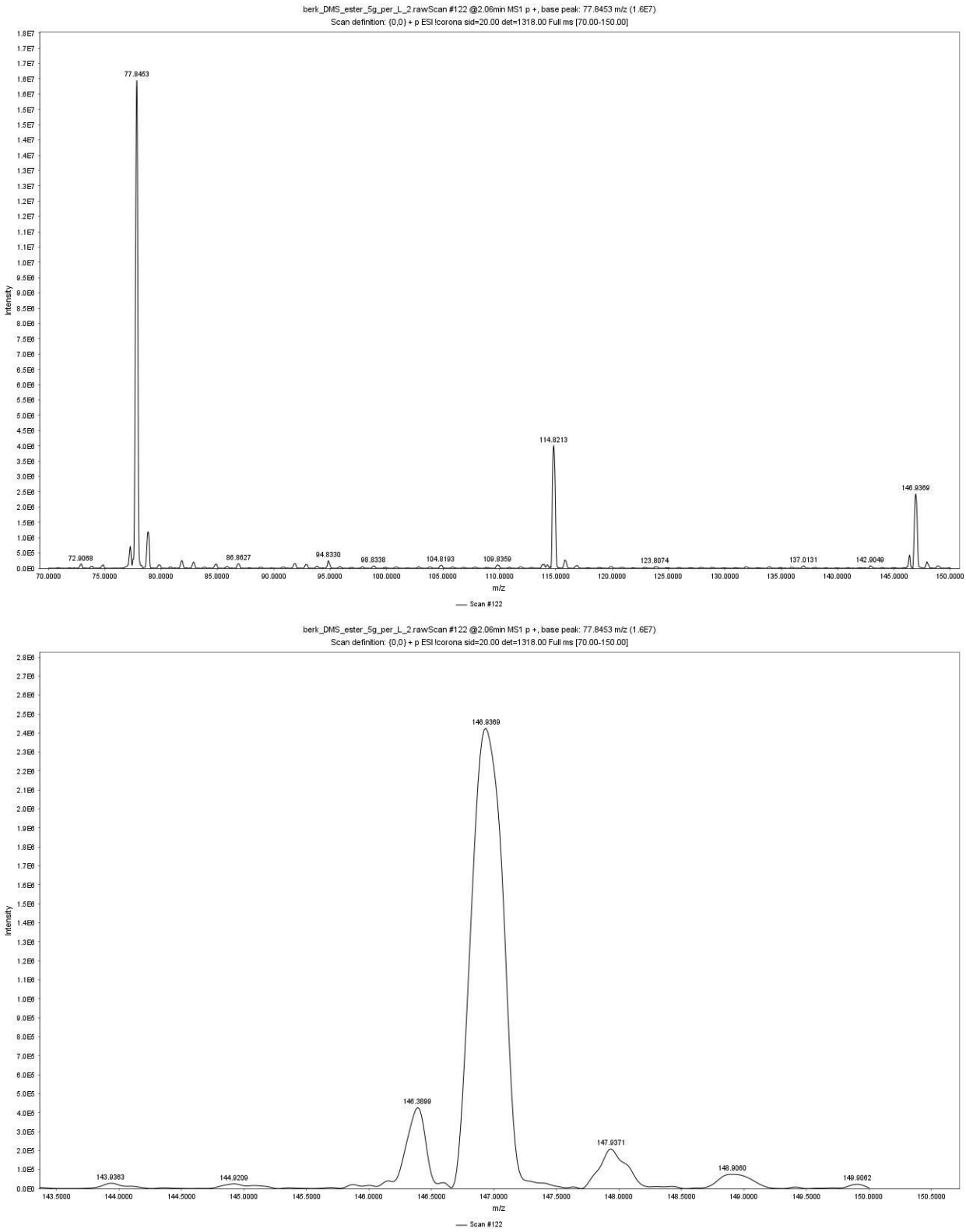
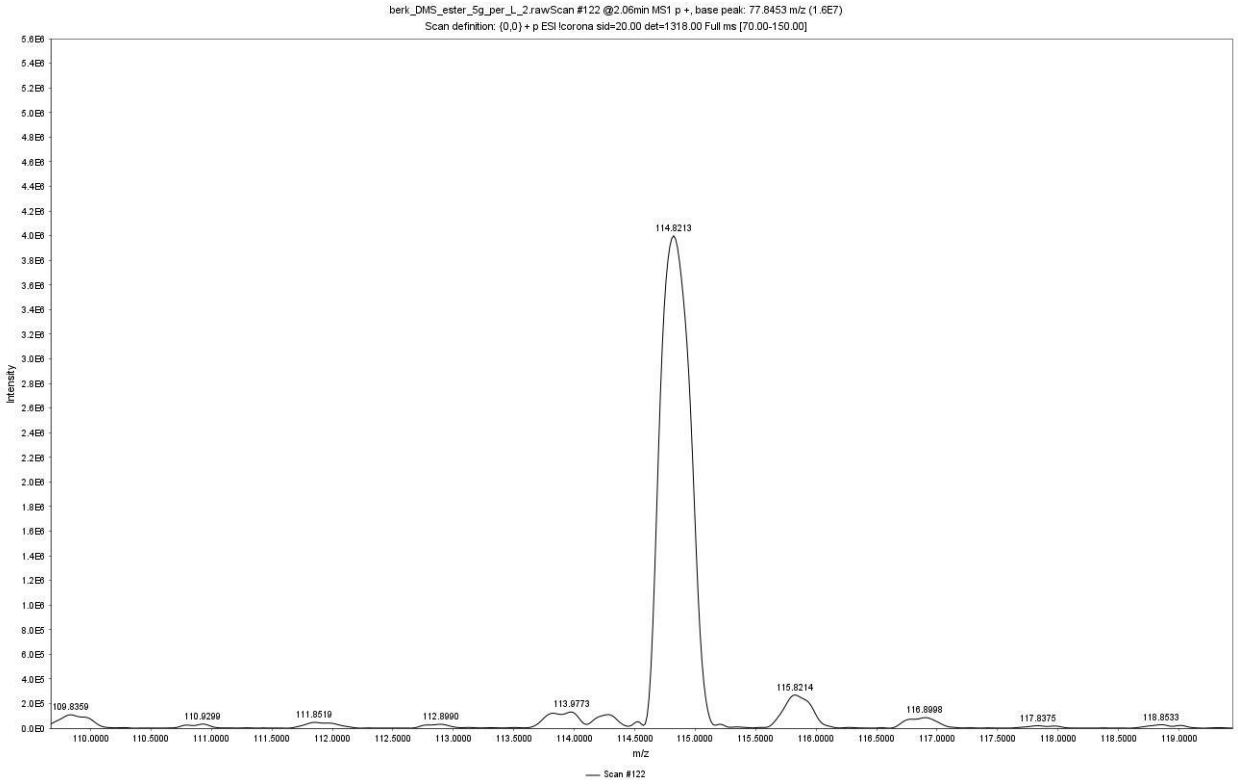


Figure 25: The first image is the spectra of 5g/L standard, second image is the magnification of the DMS peak, and the third image (below) is the magnification of the DMS fragment peak.

Figure 25 continued.



In **Figure 26** below, the time plot and spectra for injecting the 5g/L esterification standard three times in a ten-minute period. The spectra are zoomed on the DMS and its fragment peak. The 5g/L esterification standard was injected approximately at 1.28, 4.26, and 7.09min. The last image shows the time series plot of mass range 146-147m/z for the triple injection. The time difference between the first two injections when the baseline flattens was approximately 2.88min and the difference in the next two were 2.92min. From this data, it can be concluded that the duty cycle for the CIMS is around 3min.

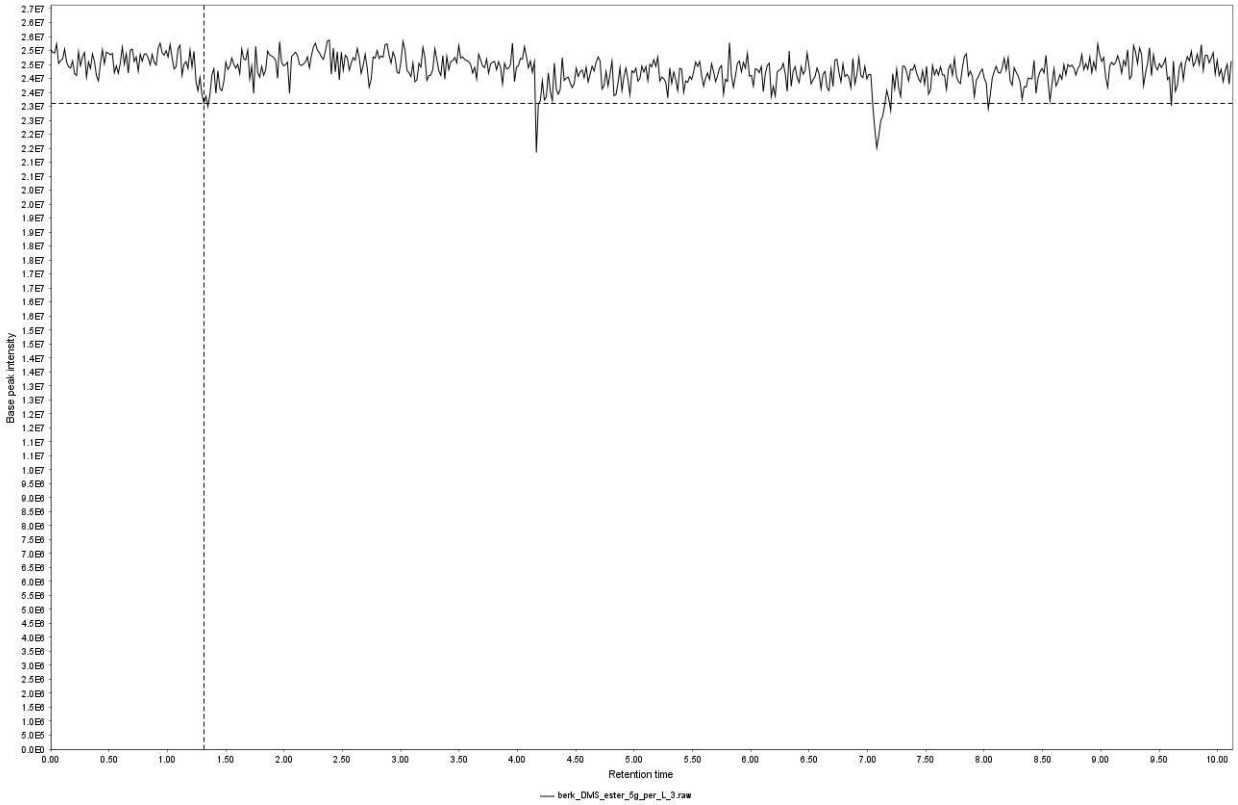


Figure 26: The first image is the time plot showing 70 to 150m/z for 5g/L SA in BG-11 esterification standard injected multiple times. The second image (below) is the spectra of the first peak from the time plot. The third image (below) is the spectra of the second peak from the time plot. The fourth image (below) is the spectra of the third peak from the time plot. The fifth image (below) is the time plot of 146 to 147m/z 5g/L SA in BG-11 esterification standard.

Figure 26 continued.

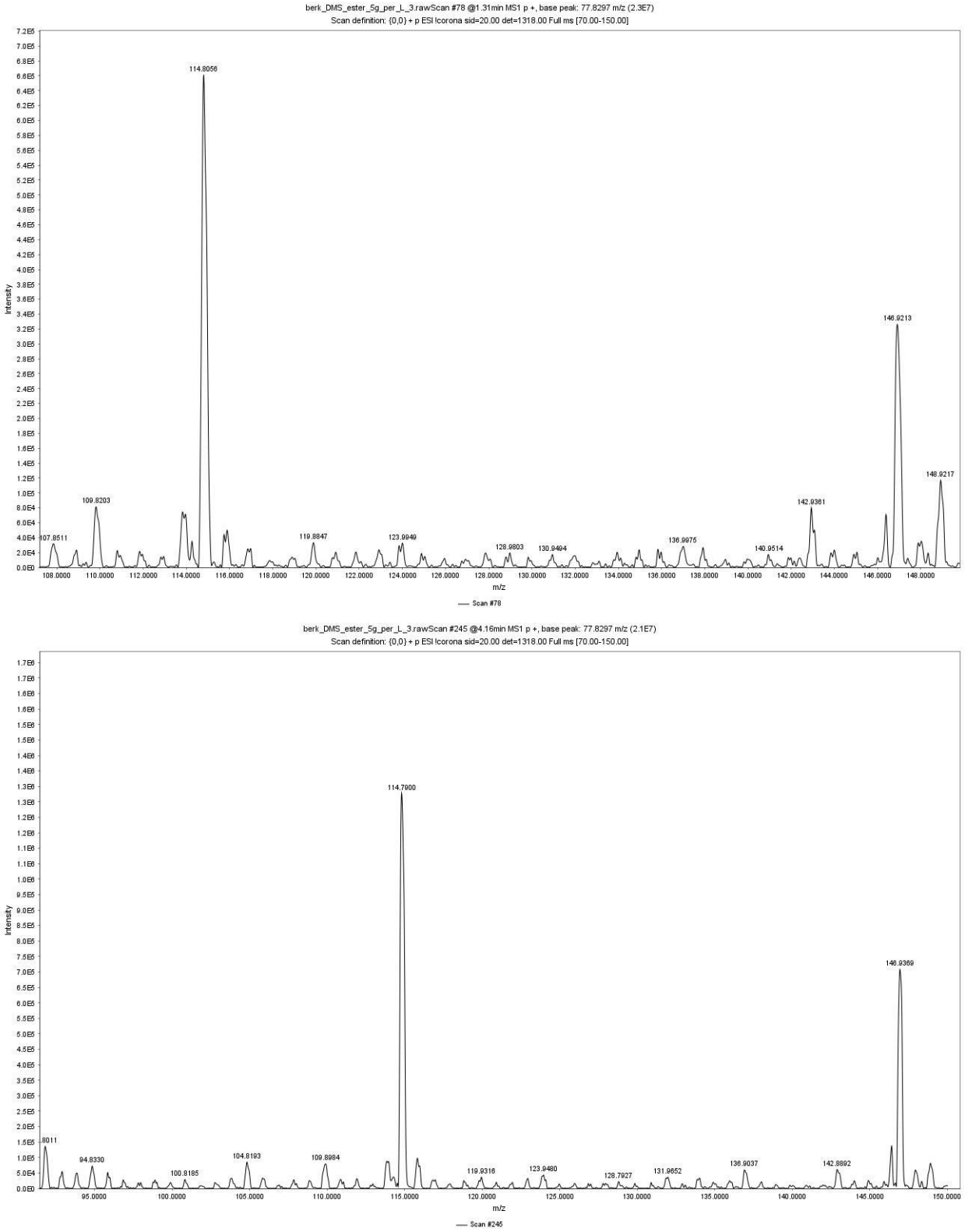
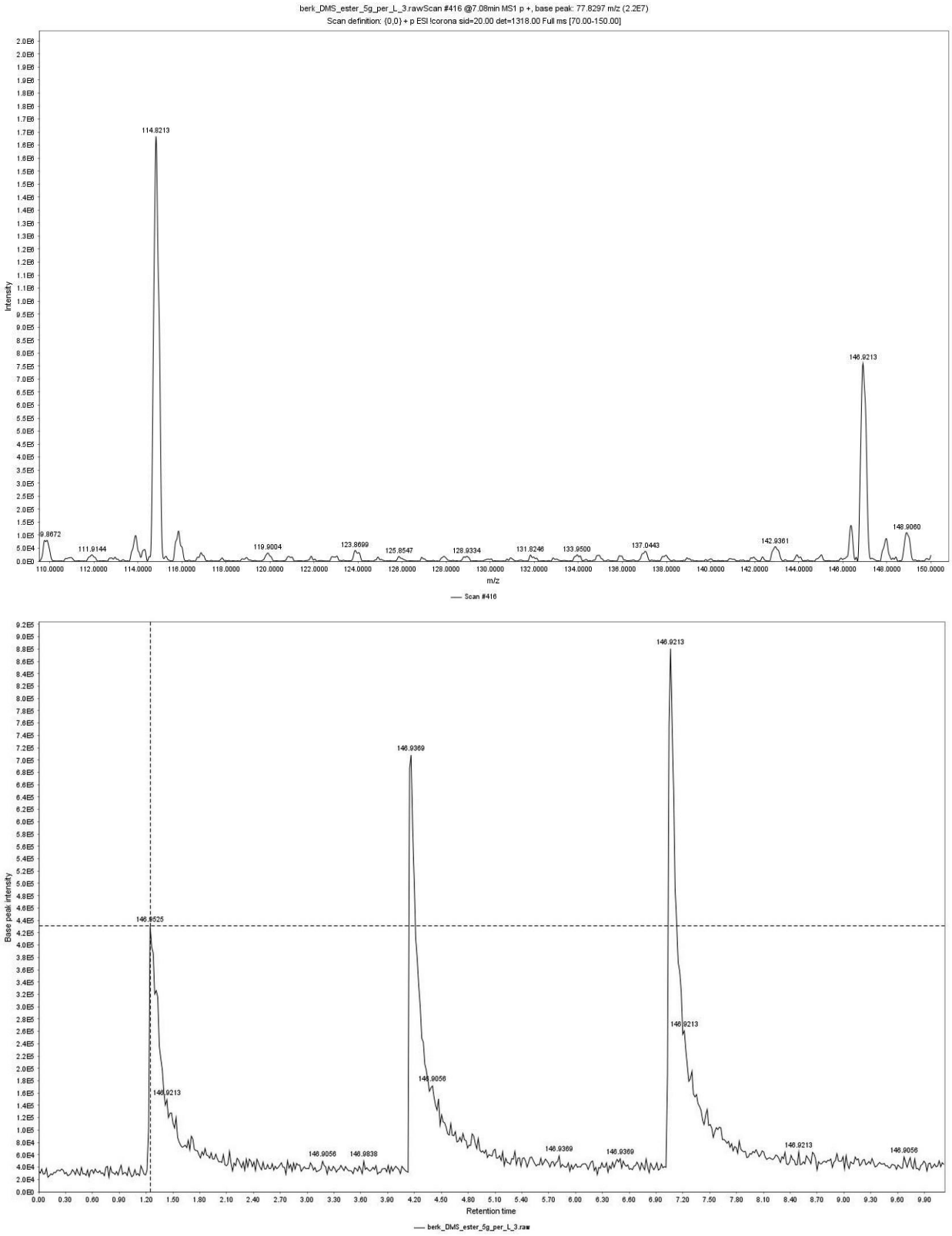


Figure 26 continued.



In **Figure 27** below, the time plots and spectra for the 5, 10, 15, and 20g/L esterification standards can be observed. The 20g/L esterification standard was recorded in a different time plot than the 5, 10, and 15g/L esterification standards.

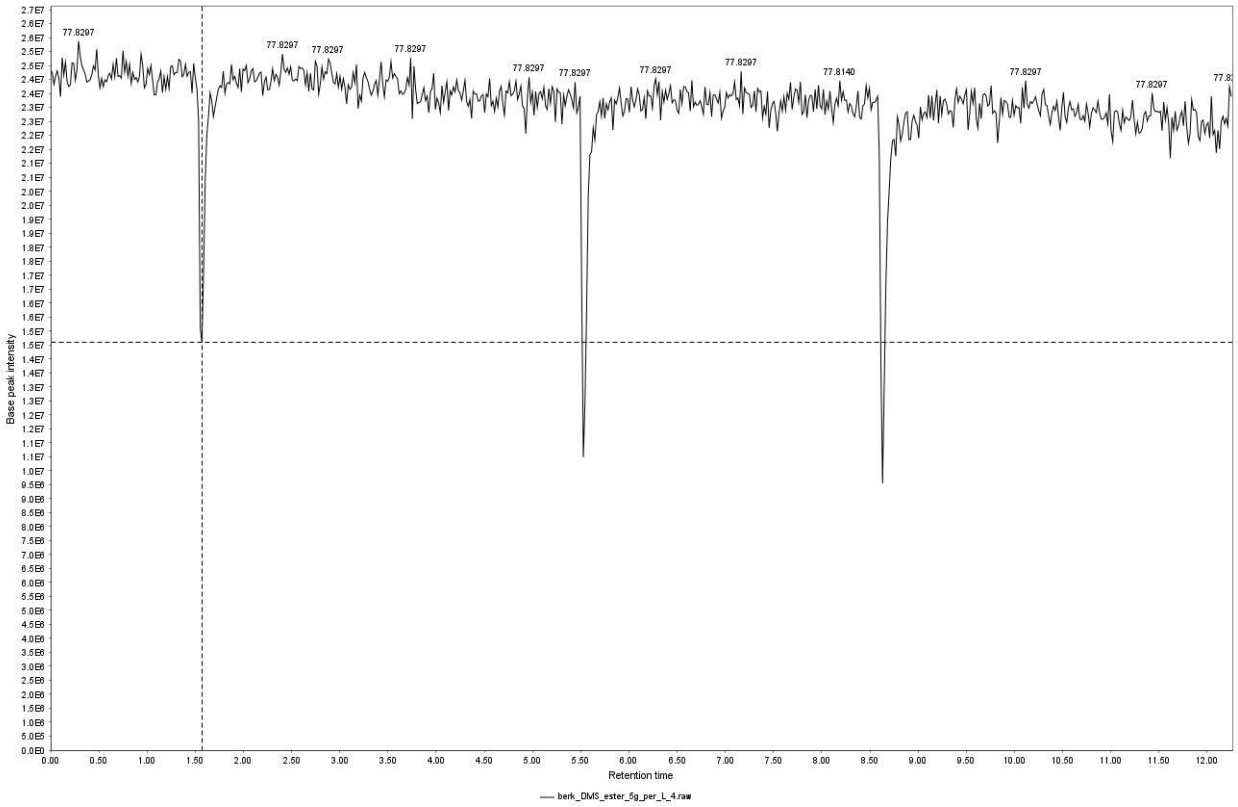


Figure 27: The first image is the time plot from 70 to 150m/z for 5, 10, and 15g/L esterification standards respective to the peaks from left to right. The second image (below) is the time plot from 70 to 150m/z for 20g/L esterification standard. The last four images (below), from top to bottom, are the spectra of 5, 10, 15, and 20g/L standards, respectively.

Figure 27 continued.

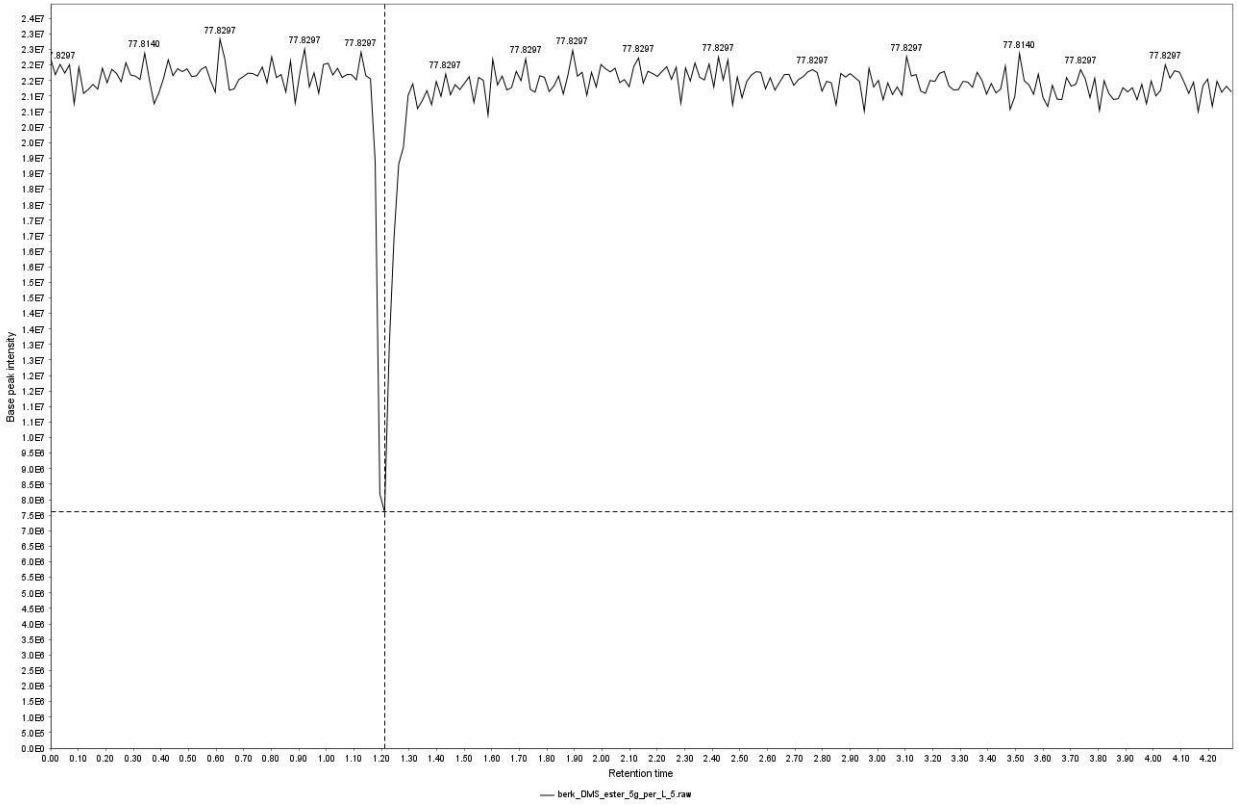


Figure 27 continued.

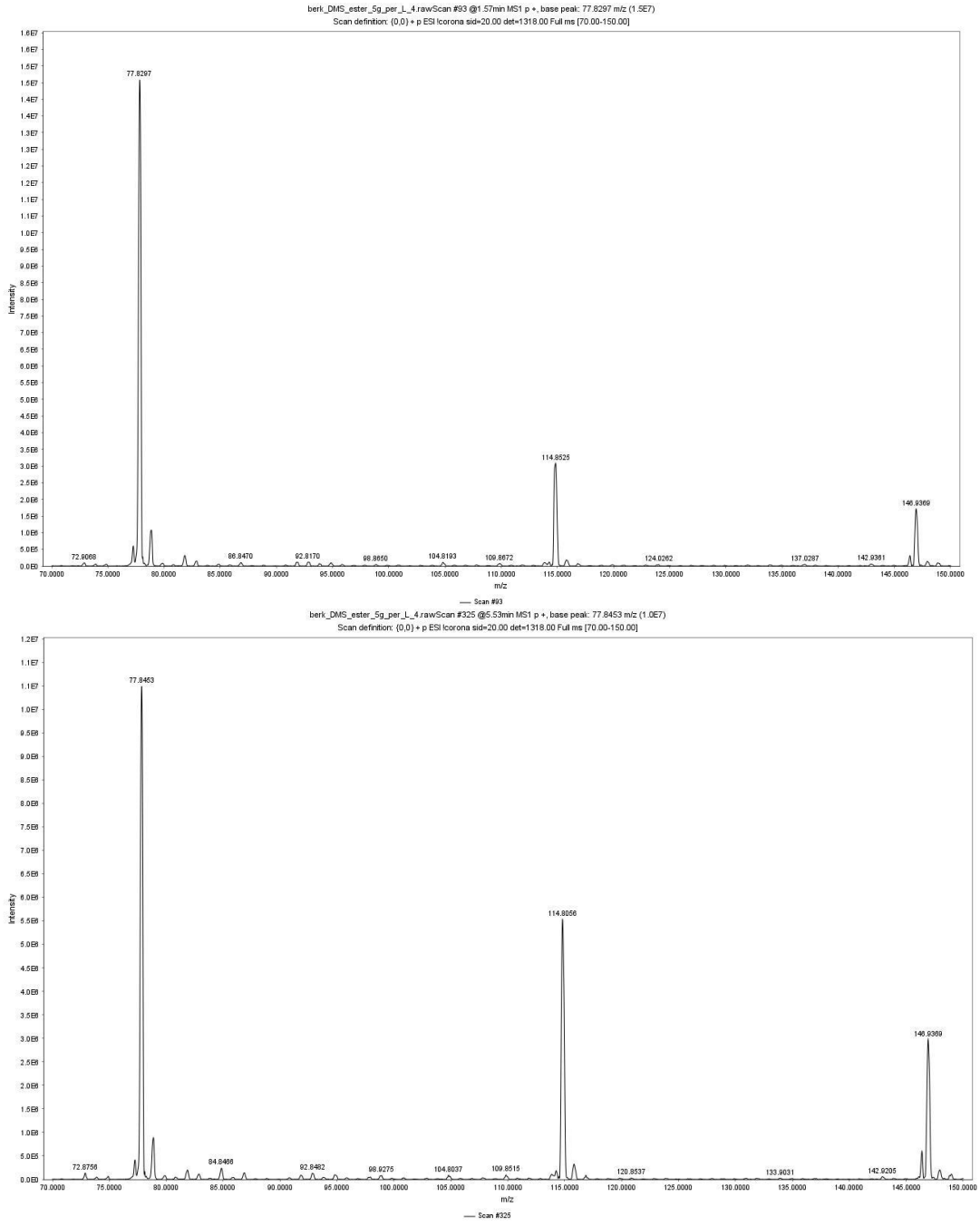
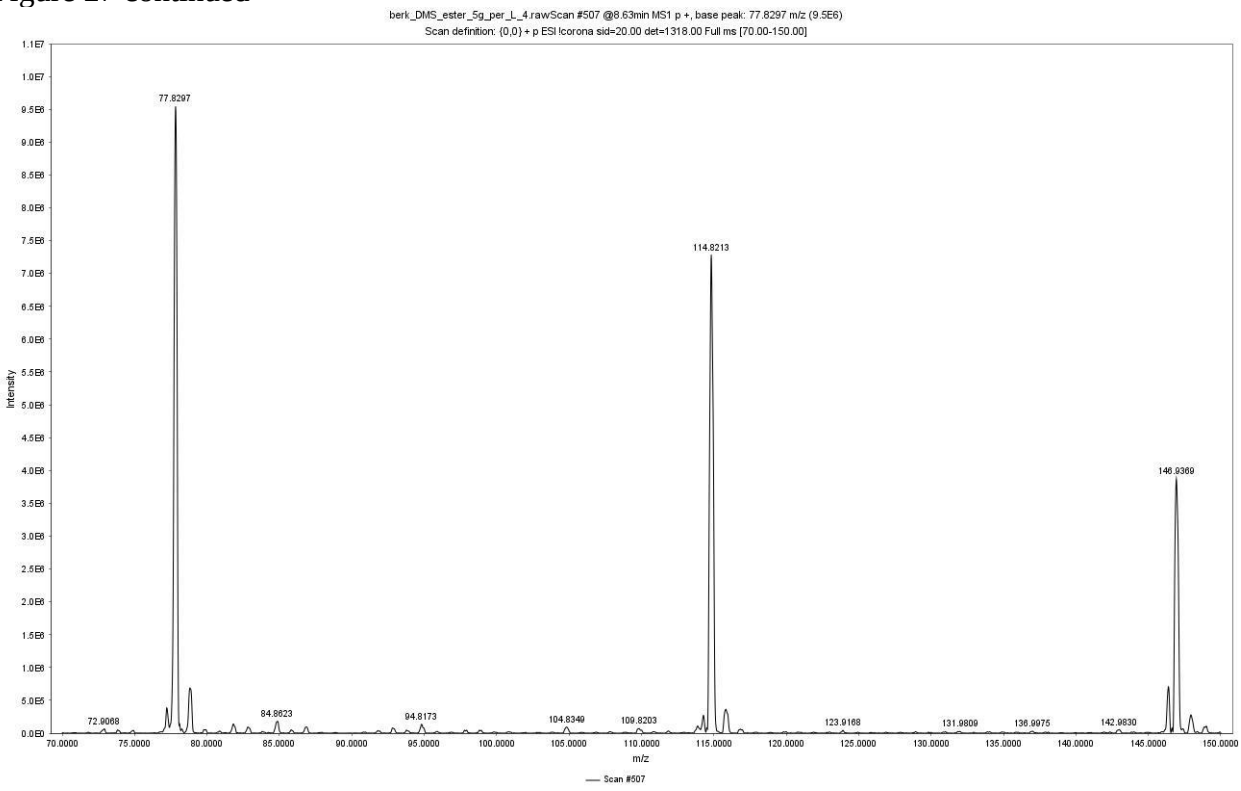
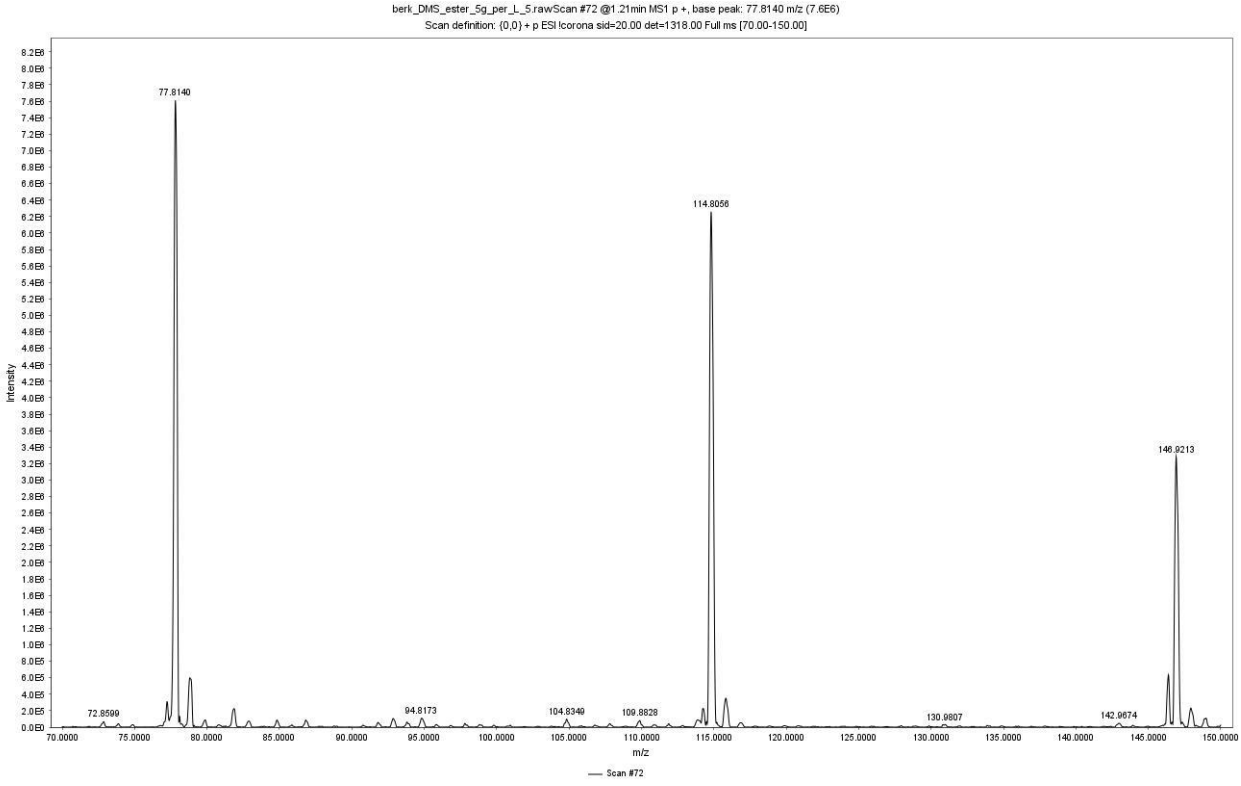


Figure 27 continued





Chapter 3 DISCUSSION

Section 3.1: pH 4 vs. pH 10 Analysis

From the data that can be observed in Section 2.2, DMS standards were prepared in two different pH solutions. One standard was prepared in pH 4 solution and the other in pH 10 solution. This was conducted to observe what type of pH solution will the DMS stay in its ester form. This finding was crucial to determine because the desire was to find a basic derivatization technique to analyze the succinic acid. One of the most basic forms of derivatization was Fischer Esterification, so it was important to discover if the pH of the solution affected the DMS compound. From the data, it was determined that, in pH 4 solution, the DMS stayed in its ester form because the DMS peak in the GCMS chromatogram did not disappear with time. For the pH 10 solution, it was observed that the DMS peak from the GCMS chromatogram started to disappear quickly after a day. Although no further analysis was done on pH 10 to determine what caused the disappearance of the DMS, because of the high pH, it was hypothesized that base-catalyzed ester hydrolysis (saponification) occurs in the solution. Since the DMS stayed as an ester in pH 4, an acidic solution, it was concluded that a Fischer Esterification could be performed on the succinic acid for derivatization.

Section 3.2: Analysis on the Crimp Vial Esterification Method

Since three of the four instruments used for this experiment required the succinic acid to be derived to its ester form, finding a faster way to prepare esterification samples was beneficial. Usually, the esterification is performed using a reflux glassware setup. If a user is given 96 samples of cyanobacteria derived succinic acid and it is required to perform esterification on all the

samples, then setting up 96 different refluxes or performing them throughout the week can be either impossible or time consuming. That is why the setup that is shown in **Figure 6** at Section 1.1 solves the issue by making the setup high throughput by the sense that it is easy to setup the glassware, the setup equipment is cheap, and multiple samples can be placed in the water bath.

Section 3.3: Analysis on the HPLC's Throughput and Cost-Effectiveness

Although the analysis of succinic acid through HPLC has been done before and has well established methods, the duration to screen for succinic acid is not the best. The data that is shown in **Figure 12** at Section 2.1 has a total run time of 17min per sample. If the user was tasked to screen 96 samples for succinic acid, then it would take 27.2hrs to screen all 96 samples. Since the HPLC has an automatic sampler, once the samples are prepared, they can just be placed on the auto-sampler and the user does not have to worry about it. However, an industrial company wants to screen and analyze many samples quickly and cheaply. It is also not cost-effective because a technician needs to be paid to prepare and run these samples daily. So, a basic 96-well plate style screening taking a little bit more than a day is not ideal for industry.

Section 3.4: Comparison of HSGC to GCMS

Both the GCMS and HSGC methods required the esterification of the succinic acid to DMS. This is because of how the GC system operates. The ester form of succinic acid is more volatile than the carboxylic acid form, hence it improves the GC analysis, and the analyte will not degrade before vaporizing. The HSGC, when compared to the GCMS, is preferable when comparing their duty cycle time and chemical work-up. The HSGC has a total runtime of 5.25min,

while the GCMS has a total runtime of approximately 5.93min. So, if a technician wanted to run 96 samples, it would take around 8.4hrs to run on the HSGC, while the on the GCMS it would be around 9.5hrs. That is approximately a 12% decrease in runtime for each day. The HSGC also does not require any chemical work-up to have the sample to be ready for screening/analysis. After the esterification reaction occurs in the hot water bath, the crimp vials just need to be dried thoroughly and placed into the HSGC auto-sampler. These benefits allow HSGC method to be a better high throughput screening method for succinic acid.

Section 3.5: Comparison of CIMS to HSGC

The screening procedure for the CIMS also required the succinic acid to be converted to dimethyl succinate. This derivatization was required because the reagent gas that could be used was benzene and DMS has a better chance of being extracted into cyclohexane than succinic acid. The reason why cyclohexane was chosen as the solvent to extract was because cyclohexane showed the least number of extraneous peaks in the spectra. When comparing the duty cycle between HSGC and CIMS, the CIMS has a faster duty cycle than HSGC. From the last image in **Figure 26**, it can be observed that the time it takes for the baseline to flatten between each injection is approximately 3min. From the same image, it can be observed that the duty cycle for the CIMS can even be brought down towards 2min rather than 3min. This would mean that, if a technician wanted to run 96 samples in day, then it would take 4.8hrs to run all the samples. This would make the CIMS the fastest instrument in screening for succinic acid. However, unlike the procedure for HSGC, CIMS does require a chemical work-up by liquid-liquid extracting the DMS into cyclohexane.

CONCLUSION

Purpose and Results

The purpose of this experiment was to compare the throughput and screening abilities of HPLC, GCMS, HSGC, and CIMS for cyanobacteria derived succinic acid. It was determined that the succinic acid stayed in its esterified form, DMS, best in a pH 4 solution. From this discovery, it was decided to use Fischer Esterification to derivatize the succinic acid to its ester form, so that it can be screened and analyzed better in instruments that needed the compound to be more volatile and/or more non-polar. Using the NIST library, provided with the GCMS, it was determined that the crimp vial method to perform esterification worked and the ester, DMS, was found to be in the gas phase (headspace) and in the liquid phase of the solution. It was observed that, if someone in industry wanted to screen for succinic acid in 96-well plate style using HPLC, which had total runtimes between 17-23min per sample, then it was not the best high throughput method and it could be improved using other instruments. It was determined that the HSGC had a shorter total runtime per sample than the GCMS and that the chemical work-up for it was practically nonexistent. CIMS ended up being the fastest instrument to use when screening for succinic acid with a total runtime of approximately 3min per sample. However, unlike HSGC, the CIMS require a chemical work-up like GCMS.

Implications for Industry

Having high throughput methods allows for industry to gather more data frequently and be more cost effective by using cheaper products and have their technicians be more efficient. The technician's efficiency factors into the cost effectiveness because the industry pays someone for the technician position. If the screening and analysis is by a high throughput method, especially if

the screening is through an automated instrument and requires an easy chemical work-up, then the technician can spend the rest of their time on other experiments/projects for the same pay they receive. Having a high throughput method also allows for industry to move towards higher well plates, such as 384-well plates, and run more samples per day.

Future Work

As a future experiment that can be conducted on succinic acid screening, a thermal desorption (TD) autosampler can be used. This TD autosampler can be attached to most GC systems, such as a GCMS. This would replace the regular ALS and, instead of using vials, a TD tube would be used. The esterification solution's liquid phase can be sampled into the tube and run through a TD-GC system. Further analysis of static equilibrium distribution formed between the liquid and the gas phases inside the HSGC crimp vials can be determined.

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