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Determination of the Structure of Sphingomyelin by Tandem Mass Spectrometry and Matrix - Assisted Laser Desorption/Ionization

Antonio Ganios

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Honors Research Project

Submitted to

The Honors College

ABSTRACT:

Mass spectrometry is a method that uses masses to determine the structure of a compound. This is completed by first charging the compound until it is a cation or anion, and then its molecular weight can be determined by measuring a mass to charge ratio. The focus of this study was determination of sphingomyelin's structure. The purpose of this was that sphingomyelin has been shown to play a critical role in many biological pathways and being able to identify this lipid could prove to be beneficial for preventative medicine. Utilizing electrospray ionization (ESI) Quadruple-Time-of-flight (Q-TOF) mass spectrometry on a Waters Synapt instrument and matrix-assisted laser desorption ionization (MALDI) TOF-TOF mass spectrometry on a Bruker UltraFlex III instrument, it was possible to verify the presence of a sphingolipid as well as the tail length and the presence of double bonds.

INTRODUCTION:

The human body is composed of various macromolecules, which include proteins, lipids, and carbohydrates. Lipids have a wide range of physiological importance and they play a crucial role in compartmentalization of cells and organelles. For example, the lipid bilayer is a regulator of cellular transport and acts as a membrane that allows necessary concentration gradients to form. Lipids are categorized by their constituents, which include fatty acid tails, a backbone structure, and head groups.

The primary focus of my project is the characterization of a sphingomyelin lipid. Sphingomyelin is the most common of the sphingolipids in mammals.[1] The typical components of sphingomyelin are phosphocholine, sphingosine, and a fatty acid as depicted in figure 1. Sphingomyelin has been shown to be involved in cellular signaling as well as membrane

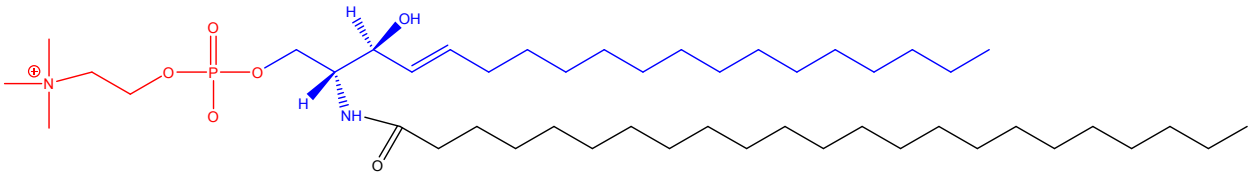


Figure 1 shows the shingomyelin lipid being analyzed. As one can see the phosphocholine is depicted in red and the sphingosine shown in blue. While the variable fatty acid tail is shown in black.

composition.[2] Typically this is done through the formation of lipid rafts composed of cholesterol, sphingomyelin, and glycosphingolipids.[1] Due to lipid rafts favorable environment for many signaling proteins such as G proteins, Src family kinases, and growth factor receptors, they are effectively able to promote or inhibit signal relays.[1]

Sphingomyelin has been shown to be involved in various other body pathways as well. Increased sphingomyelin levels are responsible for uptake of cholesterol, as the lipids often form complexes together on cell membranes.[3] Conversely, low cholesterol levels cause the body to release it from cells more rapidly. In fact, sphingomyelin plays a role in recruitment of cholesterol transporters to the plasma membrane.[1] However, excessive amounts of sphingomyelin can also be harmful. One such example is how sphingomyelin can impact insulin resistance. Studies have indicated decreasing sphingomyelin results in fewer lipid rafts, which increased insulin sensitivity.[1] One study found that by decreasing glycosphingolipids, insulin sensitivity was increased in both the liver and muscle of mice.[4] Therefore high levels of sphingomyelin could contribute to type 2 diabetes. Another concern is sphingomyelin's presence in cancer cells. More specifically it was shown that prostate and breast cancer cells had higher levels of sphingomyelin in their membranes.[5] However, another study showed that regular intake of sphingomyelin proved to decrease tumor growth for colon cancer induced in mice.[6] Further studies are needed to understand the function of sphingomyelin in regulating cellular apoptosis. Since, sphingomyelin has been shown to play an important role in many biological processes, the

ability to detect its presence and chain length are crucial to serve as a preventative health screening and potentially disease treatment.

Although mass spectrometry cannot give insight into how sphingomyelin functions in biological pathways, it does offer a method of detection at very low concentrations. It also can serve as a screening technique if further studies show certain lengths or saturations of the fatty acid tails are the source of bodily harm.

In order to detect sphingomyelin at low concentrations and with such detail, high precision instrumentation must be used. The instrumentation for this analysis will include an electrospray ionization (ESI) Q-TOF mass spectrometer and a matrix-assisted laser desorption ionization (MALDI) TOF-TOF mass spectrometer. Both of these instruments provide extremely accurate readouts with mass precision to 0.001daltons in the instruments. Both instruments require the sample to be in the gas state and under vacuum; however, their methods of ionizing are quite different. ESI initially has the compound of interest dissolved in a volatile liquid such as chloroform. The solution is then sprayed through a charged capillary in order to ionize the sample. Conversely, MALDI has the sample dried on a plate with a matrix suitable for the sample. A laser then hits the sample and causes it to be desorbed and ionized. MALDI is typically reserved for use on larger molecules including proteins, carbohydrates, and lipids.[7] Readout for both instrumentation systems is presented in the form of peaks detected based on their mass to charge ratio and abundance.

MATERIALS and METHODS:

Sphingomyelin (23:0) extracted from bovine milk was purchased in powder form (Avanti Polar Lipids, Alabaster, AL). The lipid had a variety of fatty acid chain lengths which require the use of an analytical method capable of determining these lengths. The chain distribution data that was provided by Avanti Polar Lipids can be seen in figure 2.

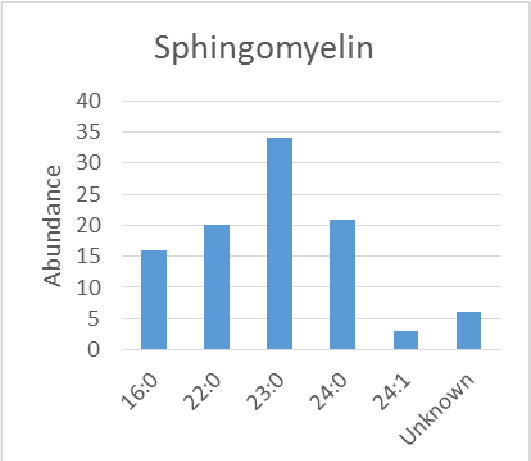


Figure 2: The following figure shows the sphingomyelin distribution as described by the Avanti Polar Lipids. Lipid in the highest concentration is (23:0). As one can see the sample was not completely pure and some unknown lipid structures were present.

ESI- Synapt:

Analysis was performed using a Waters Synapt G1 Q_TOF tandem mass spectrometer coupled with ESI. Methanol, chloroform, NaI, and LiI were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO). Samples were prepared by creating a 0.1mg/mL solution of sphingomyelin in chloroform. Samples were made in small quantities to preserve the sphingomyelin in its stable solid form. It was important to use glass vials for the preparation as plastic showed rapid lipid degradation. Lipids were then vortexed to ensure they were fully dissolved and distributed in the chloroform. In another vial, 0.1 mg/mL of NaI or LiI was created in methanol. This solution was also vortexed to ensure that the salts were fully dissolved. Samples were loaded into a 250 µL syringe. 125 µL of each the lipid and salt solution were loaded. Therefore the final concentration of the lipid being tested was 0.05 mg/mL dissolved in equal amounts of chloroform and methanol with a small amount of salt added to promote better ionization. Additional information between the differences of using NaI or LiI can be found in

the discussion. The samples were run at a flow rate of 5 µL per minute. Tandem mass spectrometry (MS/MS) experiments were performed by collisional activated dissociation (CAD) using argon as a collision gas. Samples were run primarily in positive mode because the lipid studied more readily formed cations. Data analysis was later performed using Walters' MassLynx software.

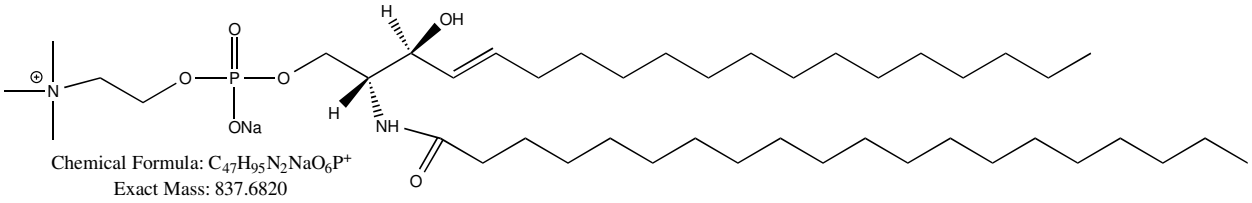
MALDI:

Analysis was performed using a Bruker UltraFlex III MALDI-TOF-TOF (tandem time-of-flight) mass spectrometer. Again chloroform, NaI, and DCTB matrix were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO). A 10 mg/mL solution of sphingomyelin was made using chloroform in a glass vial. DCTB was dissolved in methanol (20 mg/mL) and NaI in methonal (10 mg/mL). Then DCTB, the sample, and NaI salt were mixed together in the ratio of 10:5:1 respectively. This mixture was then spotted on the plate and allowed to dry. PMMA, Poly(methyl methacrylate), was used for calibration and prepared using the above procedure. Samples were then run with adjustments made to the laser power to maximize signal intensities. Data analysis was performed using Bruker's DataAnalysis software.

RESULTS/DISCUSSION:

The initial results (figure 3 in appendix) showed many peaks clustered between 600-900 Da. Examining these peaks more closely revealed a common mass difference of 14.02 Da between adjacent peaks. This is the mass of -CH_2- group and makes sense, as the sample was known to contain sphingomyelin with varying fatty acid chain lengths. The results showed a great diversity of samples found in the lipid samples ranging form sphingomyelin (24:0) to (13:0). This was surprising to see such a great variety in fatty acid lengths, but it was known that

the sample was not synthetic and therefore variations were expected. Also a cluster of peaks occurred in the 900-1000 Da range. These peaks were determined to be the lipids found in the 700- 900 Da range with the addition of a NaI. The calculated mass of sodiated sphingomyelin (23:0), the main product of the lipid sample according to supplier (shown in figure 4 is 837.6820 Da, which is very close to the mass found experimentally, 837.6807 Da. A large peak at



Chemical Formula: $C_{47}H_{95}N_2NaO_6P^+$
Exact Mass: 837.6820

Figure 4 shows how sphingomyelin appears when sodiated and shows the expected mass of the lipid. Initial experimental results show a very similar mass with a mass accuracy of 1.55 ppm.

398.2484 Da was also present, but upon further analysis this was present in many different lipid samples and likely is a byproduct of the purification process. After that tandem mass spectrometry (MS/MS) experiments were performed on the identified peaks in order to gain insight into each sphingomyelin's breakdown pathways and structure.

The 837.6701 m/z peak was then isolated and fragmented using MS/MS giving a few major peaks at 778.5999 m/z, 654.8009 m/z, and 184.0860 m/z as can be seen in figure 5 located in the appendix. Upon analysis it was determined that fragmentation would occur between different regions of the sphingomyelin head. As one can see in figure 6 one fragment is formed by loss of a partial region of the head; while figure 7 shows that the other fragment is formed by loss of the entire head group.

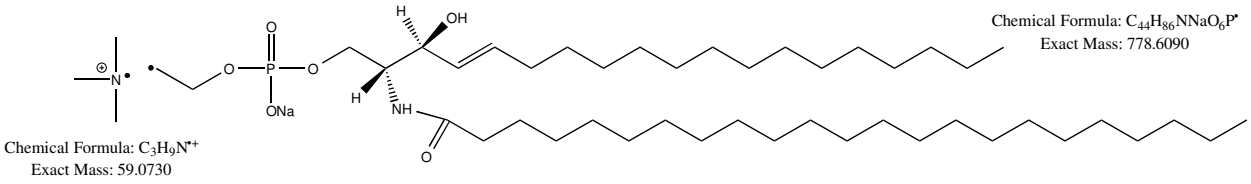


Figure 6 shows the loss of only a portion of the sphingomyelin head group.

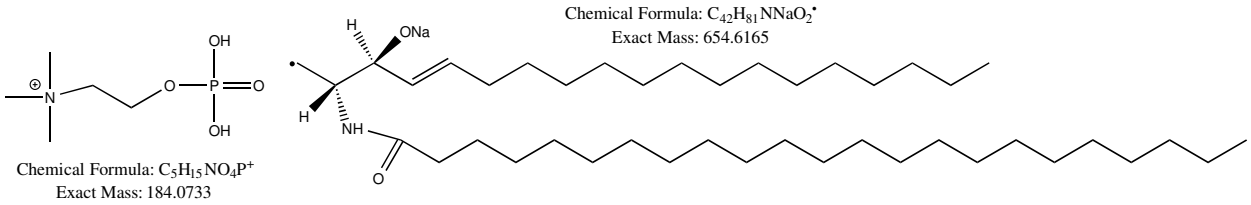


Figure 7 shows the loss of the entire sphingomyelin head group.

Likewise all the other major peaks showed similar results after MS/MS was performed, which can be seen in figures 5, 8-10 in appendix 3. At this point it was clear that regardless of the fatty acid tail length, fragmentation would occur at the head group. The experiment was performed again to verify that the trends were reproducible as seen in figure 11 in the appendix. Also, additional peaks were isolated and fragmented to see if they would react in a similar fashion. These results can be seen in figures 12-18 in appendix 3. Results were almost identical to the first set obtained showing that if conditions are consistent the lipids can be identified using these methods. Also, table 1 shows the ppm error of the major lipid peaks for all the different runs. One interesting note was that all of the MALDI data showed significantly higher error relative to the ESI data. However upon further examination one will notice that all of the experimental MALDI peaks were lower than the expected mass by roughly 0.35 Da. Therefore the MALDI did have high precision, but had low accuracy that could be corrected in future runs by a recalibration.

The next step was to use LiI salt to potentially help see additional peaks as the Li would more strongly bind to the lipid and cause it to form more fragment ions. Indeed, when LiI was added, one saw peaks with higher intensities and a few more peaks were present. This is because the lithium was able to more readily remain bound and form cations with different fragments of the sphingomyelin and these charged fragments were now detected by the Synapt. These results can be seen in figure 19-23 in appendix 3. Due to sphingomyelin not having a great deal of complexity beside its fatty acid tail, the extra fragments from using lithium as a salt were not beneficial. They did show the head group peak better (184.0733 m/z).

Finally the sphingomyelin was run on MALDI in order to see if similar results could be obtained using a different ionization method. The results were promising as peaks were detected with the characteristic 14 Da spacing as well as matching in masses. One limitation of MALDI was that it typically is used to determine masses of larger samples. Therefore results below a few hundred Da are not reliable due to interference between the matrix and sample. Therefore some of the smaller products from fragmentation were not visible such as the phosphocholine (184 Da). As one can see in figure 24 the MALDI was able to identify the different sized sphingomyelin. All together MALDI proved to be an alternative method to study the sphingomyelin.

CONCLUSION:

Overall, the experimentation proved to be insightful into how sphingomyelin's structure is broken down under stress in a gas phase. It was shown that multiple methods of mass spectrometry could be used to characterize sphingomyelin including ESI and MALDI. Also the benefits of using ESI were shown as a great amount of detail was obtains from subsequent

fragmentation. Finally, it was shown that sphingomyelin prefers to fragment between its backbone and phosphocholine head group, and that this trait could likely be used to distinguish sphingomyelin from other lipids or molecules.

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Appendix 1: Safety Considerations

Due to the nature of any lab dealing with chemicals a few safety measures must be taken regardless of the chemical used. This includes the use of gloves and safety eyewear at all times. Many of the chemicals being dealt with in the laboratory are flammable and volatile and therefore it is important to keep a clean work environment and work dealing with chemicals should be done under the hood. At the conclusion of dealing with chemicals it is important to dispose of them properly. For the chemicals used in this lab they are to all be placed under the specified waste hood and allowed to fully evaporate. At that time the vials or test tubes can be disposed or cleaned. If a spill is to occur the area should be cleared and the lab should be ventilated if the spill is significant.

Another concern in the lab is the handling of glass vials and syringes. If any glassware breaks one is to immediately clean the glassware up using a broom. Cleaning broken glassware should never be done without safety eyewear or gloves. Glass waste containers are located throughout the lab and all glass waste should be disposed there.

Finally, the mass spectrometer instrumentation is very precise and made to detect very small concentrations of samples. Therefore cleaning is often required before and after running samples to completely clean the instrumentation. Therefore it is very important that instruments are put on hold when being cleaned. Also, it is important to always wear eye protection and gloves as cleaning involves different solvents to help break down the sample residues.

Below are recommendations if an individual comes in contact with some of the most used chemicals in lab.

1) Exposure to sphingomyelin

a) If sphingomyelin is inhaled or ingested one should immediately contact a physician and the individual should be taken to an area with fresh air. If the individual feels sick emergency responders should be notified immediately to better direct the individual.
b) If sphingomyelin comes in contact with one's eyes or skin they should thoroughly clean the affected area using an eye wash station or sink for at least 15 minutes. If irritation is present a physician should be contacted.

2) Methanol
   a) If methanol is inhaled or ingested one should immediately contact a physician and the individual should be taken to an area with fresh air. If the individual feels sick emergency responders should be notified immediately to better direct the individual.
   b) If methanol comes in contact with one's eyes or skin they should thoroughly clean the affected area using an eye wash station or sink for at least 15 minutes. If irritation is present a physician should be contacted.
3) Chloroform
   a) If chloroform is inhaled or ingested one should immediately contact a physician and the individual should be taken to an area with fresh air. If the individual feels sick emergency responders should be notified immediately to better direct the individual.
   b) If chloroform comes in contact with one's eyes or skin they should thoroughly clean the affected area using an eye wash station or sink for at least 15 minutes. If irritation is present a physician should be contacted.

4) Sodium Iodide (NaI) or LiI

a) If sodium iodide is inhaled or ingested one should immediately contact a physician and the individual should be taken to an area with fresh air. If the individual feels sick emergency responders should be notified immediately to better direct the individual.
b) If sodium iodide comes in contact with one's eyes or skin they should thoroughly clean the affected area using an eye wash station or sink for at least 15 minutes. If irritation is present a physician should be contacted.

Appendix 2: Calculations

Calculating how much chloroform needs to be added in order to make a sample 0.1mg/mL was performed using the equation:

(Volume of chloroform to add) = (mass of lipid in vial in milligrams)/ (0.1 milligrams)

Example:

0.01 grams of sphingomyelin were weighed out. That is equal to 1 milligram. Therefore in order to get to a concentration of 0.1mg/mL 10 mL of chloroform must be added.

Determining the differences in mass between peaks for data was done by subtracting the peaks respective mass/charge ratios. Since only sphingomyelin was tested double and triple charged peaks were not present.

Example:

Peak 1 (m/z) – Peak 2 (m/z) = Difference in Da

Reference figure 11

$809.5700\ Da - 750.4994\ Da = 59.0706\ Da$

Calculation of PPM Error:

Observed mass: mass obtained from instrumentation

Exact mass: Theoretical mass calculated from summing the elements with consideration their respective isotopes.

$$\frac{(Observed\ mass - Exact\ mass)}{Exact\ mass}\ x\ 10^6$$

Example:

Reference Figure 5 peak A

Observed mass: 736.5580 Da

Exact mass: 736.5621 Da

$$\frac{(736.5580\ \text{Da} - 736.5621\ \text{Da})}{736.5621\ \text{Da}}\ x\ 10^6 = -5.5664\ ppm$$

$Thefore\ the\ error\ is$ 5.5664 ppm

Appendix 3: Mass Spectrometry Data

See attachment