UNIVERSITAT POLITÈCNICA DE VALÈNCIA

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Escuela Técnica Superior de Ingeniería Industrial

CHARACTERISATION OF GANGLIOSIDES IN HUMAN MILK SAMPLES AND THEIR IMPACT IN TIME OF RETENTION

Trabajo Fin de Grado

Grado en Ingeniería Química

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CURSO ACADÉMICO: 2022/2023

GDAŃSK UNIVERSITY OF TECHNOLOGY

FACULTY OF CHEMISTRY

DECLARATION regarding the diploma thesis titled:

Evaluation of the retention time of GM3 gangliosides on different types of stationary phases for reversed-phase chromatography.

DECLARATION regarding the diploma thesis titled in Polish:

Ocena czasu retencji gangliozydów GM3 z wykorzystaniem różnego typu faz stacjonarnych w odwróconym układzie faz.

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ID: 197203 Faculty: Faculty of Chemistry

Field of study: green technologies

Cycle of studies: postgraduate

Mode of study: Full-time studies

Type of the diploma thesis: engineering diploma thesis

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ABSTRACT

The field of complex lipids, specifically gangliosides, is becoming increasingly interesting to study due to the notable influence they have on the body and their presence in most human cell membranes, mainly those of the nervous system. These molecules belonging to glycosphingolipids, are composed on the one hand by a sphingosine bound to a fatty acid and on the other a chain of hydrocarbons in the presence of sialic acid, which gives the molecule the negative charge that allows differentiating GG from other lipids belonging to the glycosphingolipids. In this way, they present a wide structural diversity, giving rise to a wide range of species. Thus, all of them perform important biological functions, making their study as well as their characterization and quantification even more important. That is why in this thesis we will proceed to extract the gangliosides existing in human milk by means of a liquid-liquid extraction followed by a solid phase extraction, in order to isolate the ganglioside molecules from the rest of the molecules present in milk, such as proteins and lipids. Once the pure gangliosides have been extracted and isolated, the different species of gangliosides present in said mixture will be separated by liquid chromatography. Once the ganglioside species GM3 have been separated according to their structure and chemical composition, they will be quantified and analyzed with an HPLC column in reverse phase coupled with the mass spectrometre. In this experiment, it is intended to study the effect of the type of column used, with its respective stationary phase, on the time that the ganglioside molecules of GM3 remain retained in said column, before reaching the spectrometer detector. For this, three columns with different chemical compositions will be used, in order to study the behavior of the different GG species according to the column used. The class of gangliosides chosen for the study is GM3 as it is an exemplary class of gangliosides with a high diversity of species.

This way, a more comprehensive insight of ganglioside's behavior will be obtained.

In this way, the aim is to evaluate the retention of GM3 species on three different stationary phases used in reversed-phase chromatography. For this

purpose, the samples will be subjected to 3 experiments under the same conditions, varying only the stationary phase. Thus concluding that, given the nonpolar character of GG molecules, which varies from one species to another according to their fatty acid chain length and number of unsaturations, the more hydrophobic the molecule, the shorter the retention time due to the lower affinity and interaction between the two.

TABLE OF CONTENTS

ABBREVIATIONS

ESI	Electrospray ionization
GG	Ganglioside
LC	Liquid chromatography
LC-MS	Liquid chromatography mass spectrometry
LMSD	Lipid map structure database
m/z	Mass-to-charge-ratio
MS	Mass spectrometry
RT	Retention time
SPE	Solid phase extraction
HPLC	High-Performance Liquid Chromatography

INTRODUCTION AND AIM OF THE STUDY

Gangliosides are amphipathic molecules belonging to the glycosphingolipid (GSL) family. This amphipathic character is determined by the composition of said gangliosides, which are formed by a chain of fatty acids of variable length linked by an amide bond to a sphingosine. Thus, there are many different species of gangliosides which are differentiated by the length of the fatty acid chain and the number of unsaturations, double bonds, present in their structure.

What differentiates GG from the rest of GSL is the existence of at least one sialic acid in its structure. This is a monosaccharide made up of 9 carbon atoms and which is located mainly at the ends of oligosaccharides, glycoproteins and glycolipids. The order of the sugars and the type of glycosidic bond in each ganglioside give rise to a wide variety of them.

The most common ganglioside in nerve tissue, including the brain and peripheral nerves, and the one to be analyzed in this thesis, is GM3, whose oligosaccharide contains the following residue union: Glc-Gal-Neu5Ac [1]

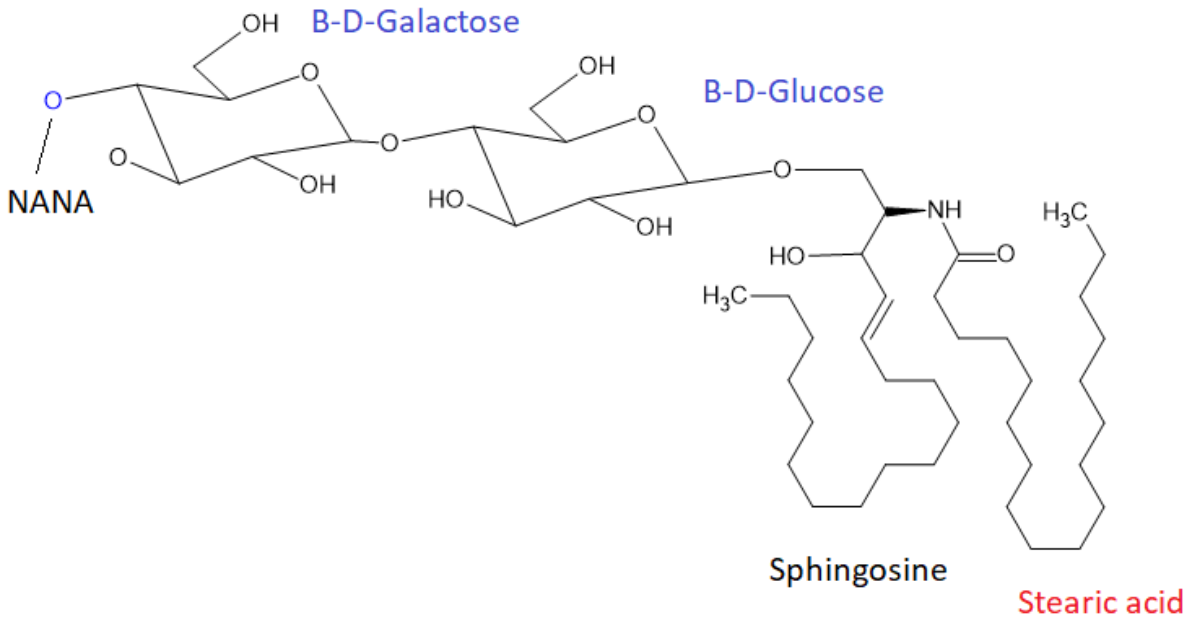

Figure 1. Structure of a ganglioside GM3 molecule.

In human milk, gangliosides are mainly found in the milk fat globule membrane (MFGM), where the polar part of the molecule, the oligosaccharide, interacts with the outside, while the lipid chain (ceramide) remains attached to the membrane and acts as an intracellular regulator. This is why breast milk regulates the immune, metabolic and microflora systems of infants who ingest it, having a great impact on the absorption of nutrients and the correct functioning of the infant gastrointestinal system. It is, therefore, important to study the structures of the gangliosides found in milk in order to determine their functions in the organism and their physiological effects [2].

The principal aim of the experiment is to study the retention of gangliosides GM3 on reversed-phase columns with the different type of stationary phase. In this way, chromatographic technique will be used to separate the different species of gangliosides existing in the mixture that makes up the sample to be analyzed, according to their chemical structure. Once the different gangliosides have been separated according to their composition, the affinity of each of them with the stationary phase will be studied, according to their chemical characteristics. This way, depending on the column used for the chromatographic separation, and its hydrophobic properties, there will be a certain affinity between the GM3 molecule and the stationary phase that will cause it to remain retained in the column for more or less time, before reaching the spectrometer detector. In other words, depending on the chemical composition of the stationary phase used, the inter or intramolecular forces existing in it, as well as the polarity that is present according to its structure, each species of GG will present a different retention time. Therefore, not only it is intended to analyze the variations caused by the different structure of certain selected gangliosides, but it is also intended to examine the effect that the use of one chromatographic column or another has on the same ganglioside, with its respective stationary phase. That is why three selected columns will be used; silica gel modified with C18 groups (Biozen Oligo C18), biphenyl and pentafluorophenyl (F5) groups, with the same chromatographic conditions but different chemical composition, in order to compare the retention time results obtained for the same ganglioside in each of the columns. In this way it will be possible to determine which type of column they are more related to and

whether there is more or less interaction between the ganglioside molecule and the stationary phase, in order to know which type of column should be used according to the time it is desired that it takes the ganglioside to reach the detector of the spectrometer. It should also be noted that inside GG GM3 class, there are different ganglioside species depending on the structure they present in the fatty acid chain, both in terms of their length and the existence of double bonds, which will influence the structure of the molecule and, therefore, its polarity. Thus, each ganglioside species will have a different interaction with the stationary phase or will be more or less related depending on its composition. This is why it is important to separate the gangliosides that are mixed in the initial sample into different species in order to carry out a correct analysis, since each one will have a different behavior in the chromatographic column.

THEORETICAL PART

1. Gangliosides - chemical structure, biological functions and classification

It is well known gangliosides are present in most human tissues, specially in the central nervous system, and play an important role in nervous system regulation and in other biological functions such as human cell evolution, cell signaling, identification and growth as well as they contribute to tissue differentiation and photosynthesis. [3] They are of great importance in human development as they act as receptors which stimulate communication between cells in the nervous system and, therefore, due to this importance, they have been chosen as the object of study in the following thesis. They are not only present in human tissues but also in some biological fluids such as animal milk. Therefore, gangliosides used for the study will be extracted from the milk.

As a matter of fact, gangliosides are complex lipid molecules present in the plasma membrane of several types of human cells. They are called gangliosides because of their abundance in ganglion cells. Moreover, they mainly make up the plasmatic membrane of nervous system cells, where they constitute around 10-12% of the lipid tissue and are mostly abundant in the brain [4]. Its structure consists of a large polar head, a ceramide base and up to four sialic acids. The simultaneous presence of hydrophobic and hydrophilic groups in the structure of these molecules conferes them an amphipathic character [5]. Moreover, not all GG´s present the same structure. Depending on the number of double bonds, the sphingosine's length, the fatty acid substituent, the number of sialic acids, as well as the presence of isomers in their structure, gangliosides can be classified into different species, each of which present a different chemical structure. Thereby, different species of gangliosides will be designated with an abbreviation, which will vary depending on the characteristics of its structure. Nomenclature is as follows:

- First of all, a G, making reference to the fact the molecule belongs to the lipidic groups of gangliosides.

- The second initial is either an M, D, T or Q, depending on the number of sialic acid residuals. An M corresponds to monosialoganglioside, only one sialic acid waste, and most common GG´s classified in this subgroup are GM1, GM2 and GM3. A D in the second initial refers to disialoganglioside, such as GD1a, GD1b, GD2 and GD3, which contain two sialic acid residuals. And, at last, a T makes reference to trisialogangliosides with three residuals, such as GT1a and GT1b.

- The last initial is a number which makes reference to the succession of molecules attached to the ceramide base.

- Letters a, b or c define the position of the sialic acid. [6]

Depending on the class they belong to, each ganglioside will present a different function.

Thereby, GG's can be classified inside the heterogeneous group of organic compounds known as lipids. Gangliosides in particular belong to the group of sphingolipids, specifically to the neutral subgroup glycosphingolipids, which are lipids with a sphingosine structural base, according to the classification established by the LipidMaps website [7].

Moreover, sphingolipids are complex lipids which derive from the 18-carbon unsaturated amino alcohol sphingosine. They are said to be the second more abundant lipid group and are mostly found in the membrane of cells.

Therefore, GG´s can be defined as very large polar heads with a ceramide base, conformed by the before mentioned ceramide (fatty acid, which can be saturated or monosaturated, + sphingosine), an oligosaccharide and at least one sialic acid. It is the presence of these acids (N-acetylneuraminic) that differentiate them from other glycosphingolipids. They belong to the saccharides group and tend to incorporate to proteins or lipids during the glycosylation process which takes part in cells. Furthermore, they are

negatively charged acids which provide cells with positive charge and contribute to intracellular signaling.

Besides, sphingolipids are a particular group of lipids essential in the plasma cell composition of many cell types in the body. Thus, they are responsible for a wide variety of cellular functions, from cell-cell interaction, proliferation, migration, adhesion to cell signaling [8]. They are formed by the adhesion of different substituents to the alcohol of the ceramide. Depending on if the substituent contains lipids or not, two groups can be distinguished: phosphosphingolipids and glycosphingolipids. Gangliosides belong to this second group of lipids and are mostly found in the nervous system, on the external membrane of cells.

Therefore, it could be said gangliosides are amphipathic complex molecules as they present both hydrophobic, due to their content in lipids (fatty acids), and hydrophilic behavior, due to the presence of carbohydrates (saccharides).

As it has been said before, analysis of gangliosides composition is going to be exposed in this thesis. On that account, it is wanted to analyze the structure they present in human milk, the different subtypes there are depending on the length of the fatty acid chain and how this affects to the retention time they spend in the chromatographic column until they reach the detector in the mass spectrometer. No matter how, obtaining a high concentration of human milk gangliosides is quite difficult. That being the case, another commercially available standard, from bovine milk samples, was the one injected in the MS in order to be able to run the experiment. This is possible because both types of milk present a similar and, therefore valid composition for the analysis.

1.1. Gangliosides in human milk (GM3 and GD3 specifically)

Although it is only going to be analyzed one type of ganglioside, GM3, and its subtypes, it should be taken into consideration other important types of GG´s.

Consequently, it has been observed that the most common gangliosides present in human human milk are GM3 (N-acetylneuraminic acid) and GD3 (NANA), whose concentration varies during the lactation process. For example, during the first phases, in which human milk is defined as colostrum, milk is highly concentrated (it oscillates between 20-100 mg/liter) and, therefore, it is rich in proteins and in nutrients. It contains a high amount of nutritional and immunological components and lipids, within which it is worth noting the presence of ganglioside GM3, which are necessary for newly born child's development. Based on studies carried out in laboratories by HPLC-MS devices, it is possible to determine an approximation of the ganglioside content of human milk. The results of these studies indicated that, as was well suspected, GD3 is an abundant molecule in milk. Thus, if the mean gangliosides present in the human milk produced is 6.33 mg/day, 3.02 mg/day correspond to the GM3 species and 1.51 mg/day to the GD3 species, also notably present but to a lesser extent [9]. However, these concentrations vary as the lactation process progresses.

As the lactation process proceeds and the colostrum phase comes to an end, human milk starts to be produced regularly. This happens around two weeks after the birth and causes a decrease in the concentration of GM3 gangliosides.

The same happens with GG´s GD3´s concentration during the lactation phase. However, GD3´s concentration in human milk (1-20 mg/mL) is relatively low in comparison with GM3´s (20-150 mg/mL). In other words, the content of GD3 decreases while the content of GM3 increases. [10]

Nevertheless, it is important to mention that the composition of human milk presents a dynamic and variable character since it depends on each woman and the external conditions that involve her.

Although both of them are gangliosides and perform important biological functions, they differ from each other in the number of terminal sialic acids. GD3 is formed by one terminal acid whereas GM3 by two. In regards to GM3, it is present in great abundance in the brain and the liver. This is due to the absence of a biosynthetic enzyme whose function is to inhibit the formation of ganglioside GM2, which is expressed by a variety of human cancer cells, as from GM3. However, GM3 is the cause of hereditary diseases which involve metabolism and originates an excessive storage of sphingolipids in the cytoplasm.

On the other hand, GD3, it is located in the golgi apparatus and arises from the union of a sialic acid residue to its precursor, the ganglioside GM3 [11]. Moreover, it is expressed as tumors, neuroblastoms, sarcoma and leukemia and presents a high capacity to accumulate due to the activation of CD95, an antigen of the tumor necrosis factor receptor family. Accordingly, in order to prevent the accumulation of ganglioside GD3 and, therefore, the negative effects it entails GD3 synthase should be suppressed, blocking this way CD95´s activity and the cellular death caused by the ganglioside´s ceramide.

What's more, the intracellular accumulation of GD3 in mammalian tissues contributes to mitochondrial damage and, although its presence is less frequent than that of the GM3 species, its expression is multiplied in the face of pathological conditions such as cancer or neurodegenerative disorders, promoting its multiplicity [12].

Therefore, as it has been said, gangliosides present the ability of modulating the immune system, so, as they are present in mammalian milk, and their content and distribution vary during lactation, as has been explained above. Since this milk with variable ganglioside composition is given orally to newborns, it can be concluded that gangliosides do influence the development of newborns' organs and can cause alterations in the newborns, mainly in the intestine and its microflora [13].

Nevertheless, human milk is composed of various components such as lipids, proteins, sugars and oligoelements, which makes it difficult to identify

and study the composition of gangliosides. They are little abundant and need to be isolated from proteins and lipids in order to carry out a proper study and an adequate analysis in the MS.

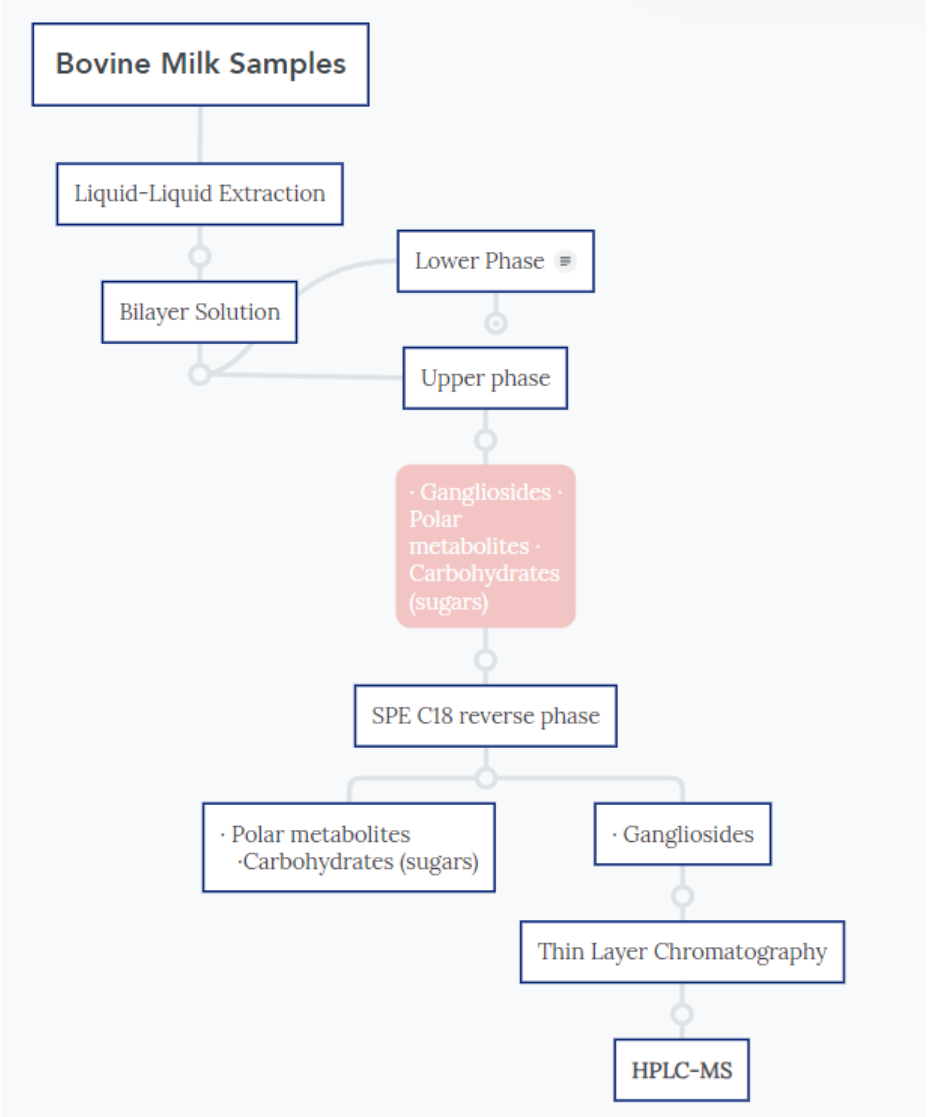

Figure 2. Scheme of the experimental procedure typically carried out from sampling to analysis of isolated gangliosides.

2. Analytical techniques used in gangliosides analysis in human milk samples.

In regards to the techniques implemented for gangliosides analysis with the use of mass spectrometer, first of all, there will be highlighted two sample preparation techniques, in order to obtain the GG isolated: liquid-liquid extraction (LLE) and solid phase extraction (SPE). This way gangliosides are extracted by chloroform – methanol – water mixture, obtaining as a result of this extraction a bilayer solution. The upper layer will contain the gangliosides mixed with some impurity of polar lipid subclasses and some remaining protein, which will be duly removed in a subsequent C18 solid phase extraction (SPE) [14]. The lower layer will be discarded since it does not contain any ganglioside molecules, which are the species of interest.

In milk samples, gangliosides are mainly found in the milk fat globule membrane (MFGM) and, due to the complexities of the molecules containing them, analysis requires complex sample preparation and subsequent gas chromatography and mass spectrometer analysis.

This way, this first method is a widely used technique of sample separation which consists in the separation of components which are initially mixed in the same sample, striking a balance between them and creating two layers in the presence of a selected solvent. This transfer of components will take place depending on the affinity they have with the chosen solvent. Therefore, for this to be possible, the molecules which want to be separated must be immiscible with each other, and only one of them can be soluble in the solvent, obtaining two phases as a result: the upper phase and the lower phase.

In the upper phase we will find the gangliosides, polar metabolites and carbohydrates (sugars) and in the lower phase we will have lipids. Proteins are precipitated between phases.

As it has been mentioned before, this first LLE extraction is not 100% effective. As GG contain a large amount of polar lipids, due to their polar head, some phospholipids are transferred to the upper phase and may obstruct the signal emitted by the MS. If not removed this set of proteins and lipids from the

upper phase, they could hinder the analysis. This is why the tubes containing gangliosides after evaporation (upper phase) will subsequently be subjected to a solid phase extraction with C18 in reverse phase, in order to culminate with the purification and increase the effectiveness of the analysis. The principal objective of this second extraction, SPE, is, therefore, the isolation of analytes from a complex matrix.

This will be implemented only to the upper phase which is the one containing our molecules of interest, GG.

This technique is quite eco-friendly, as it allows the use of greener organic solvents in sample treatment [15].

It also presents several advantages such as ease and speed, as well as the reduced amount of solvents required for its development.

There is a diverse set of phases for SPE, which base their separation on ionic, hydrophobic or polar interactions between the sample components.

An example of the development of this technique is the introduction of a solvent into the cartridge in order to wet the sorbent and the subsequent introduction of the sample containing the analyte where it will be filtered through the solid phase, with the analyte, GG, and some impurities being retained on the sorbent. The sorbent is then washed to remove impurities and the analyte is collected.

In order to select the most suitable SPE stationary phase, physico-chemical parameters such as pH, pKa and solubility of our analytes have to be taken into account. Thus, there are different types of phases: reverse phase, normal phase, ion exchange and mixed mode, which combines normal and reverse phase.

In the case of reverse phase, the retention mechanism is based on non-polar (hydrophobic) interactions, i.e. dispersion forces or Van der Waals. [16]

In contrast, the normal phase retention mechanism is based on polar interactions such as hydrogen bonding, pi-pi bonding, dipole-dipole, and induced dipole-dipole.

Another possibility for the stationary phase is the ion exchange phase. This is based on the electrostatic attraction between the charged analyte and the functional groups with oppositely charged sorbent functional groups. The selection of this stationary phase is mainly due to the fact that it accepts both aqueous and organic samples (mixed mode phases).

Thus, a commonly used stationary phase in HPLC columns is silica. This has a high affinity with both polar and apolar compounds, so it has a high retention capacity for the lipid chain and sialic acids, thus remaining gangliosides retained on the silica column, while undesired components such as proteins and other lipids are washed and removed with the appropriate solvents. Subsequently, the gangliosides are removed from the column using more polar solvents.

Regarding the solvents to be used, these will depend on two main factors: the polarity and solubility of the analytes. Thus, in reverse phase, non-polar solvents will be used, while in normal phase, polar solvents are required.

After applying SPE to our samples of the upper phase, it is observed that the extracted gangliosides are of the GM3 type as they only present one NANA.

2.1 Techniques used for the separation of gangliosides- RP and HILIC, MS

2.1.1 Thin layer chromatography

Regarding thin layer chromatography (TLC), we will highlight that this is a method, both quantitative and qualitative, used for the separation of the different compounds that make up a mixture due to the differences in affinity that these present with the chosen stationary phase.

In this case, the stationary phase consists of a thin layer of adsorbent material, which is usually silica gel, which covers an inert plate surface, such as aluminum.

In this way, a small quantity of the sample is deposited, or punctured, on the surface of the plate, which is then introduced into a container with a mobile phase, an organic solvent. The mobile phase presents a non-polar character, contrary to that of the stationary phase, since silica is a polar material.

Depending on the polarity of the compounds that make up the sample, they will have a greater affinity with the stationary phase, being very little dragged by the non-polar mobile phase or, on the contrary, they will be very similar to said mobile phase, interacting very little with the stationary and being dragged by the mobile more easily. For this reason, the most polar components of the mixture will present a high interaction with the polar stationary phase, which is why they will be the last to elute, while the less polar ones will be very easily carried away by the organic phase given their low interaction with the organic stationary phase, so they will be the first to elute.

When the solvent has completely covered the plate, it is removed from the container and dried. Components already separated appear as dots on the board. It can be determined to which compound each point belongs by the retention factor (RF) of each component.

The value of this quotient will be higher for nonpolar compounds and lower for polar ones, since they tend to move more slowly. [17]

In the case of ganglioside analysis, this technique is usually preceded by a purification process thereof by means of solid-liquid extraction and SPE. Once the ganglioside fraction of the sample has been isolated, it is separated by chromatography.

As for the stationary phase, most commonly in this case it is, as has been indicated previously, an aluminum plate covered by a thin layer of silica gel. The silica gives the stationary phase a polar character that will allow the gangliosides to be separated according to their polarity and their interaction with said hydrophilic phase.

The ganglioside sample will be punctured on the plate through a microcapillary tube.

The solvent that will be required for the mobile phase will depend on the degree of separation that is desired to obtain based on the polarity of the GGs.

The TLC plate can be submerged in a dye solution in order to stain the different dots that have formed as a result of bathing the plate in the solution and thus make it easier to visualize.

However, even though this technique has various advantages given its simplicity and low cost, the use of other chromatographic techniques for the separation of these molecules, such as HPLC-MS, is recommended.

2.1.2 High performance chromatography coupled to mass spectrometry

In order to identify and characterize different ganglioside species present in the extract of a human milk sample the hydrophilic interaction liquid chromatography (HILIC) coupled to a negative-ion electrospray ionization tandem mass spectrometry can be used. [18]

In regards to HILIC, Hydrophilic interaction liquid chromatography it is the most suitable technique for the retention and separation of polar compounds. This technique is, in turn, known as reverse reverse phase, since it works contrary to conventional reverse phase chromatography. Thus, the stationary

phase is polar and the aqueous part of the mobile phase, which is organic in nature, constitutes the strongest solvent.

Thus, this aqueous part adsorbs on the surface of the polar stationary phase, forming a layer. It is in this layer where the polar analytes are adsorbed as a consequence of the hydrogen bonds between the polar functional groups and the stationary phase, as well as by the electrostatic interactions between the ionized groups.

Since the operation of this technique is the opposite of that of a reverse phase chromatography, the order of elution will also be the opposite.

The most hydrophilic compounds are those that present a greater affinity with the polar stationary phase and, therefore, hydrophilic, and for this reason they will be retained for a longer time and will elute later. The non-polar compounds, that is, the less hydrophilic ones, will hardly interact with the stationary phase, so they will pass through the column more easily than the polar ones and will elute sooner.

As for gangliosides, they can be separated by this technique thanks to the hydrophilic nature that the presence of polar groups, such as sialic acids, gives them in their structure.

The gangliosides, after having been isolated from human milk samples using the above techniques, the pure gangliosides are dissolved in a 2:8 mixture of water and methanol, before being able to be injected into the HILIC column, of hydrophilic stationary phase, being the silica column the most used.

In this way, the different types of gangliosides are separated according to the hydrophilicity of their structure. The less hydrophilic will elute earlier and the more hydrophilic later.

To carry out the separation of the gangliosides, however, the HPLC technique was carried out in reverse phase, where the stationary phase used is nonpolar and the mobile phase is polar or, at least, much more polar than the stationary one. Thus, the gangliosides that present a greater interaction with

the stationary phase used will be those that present higher lipophilicity in their structure. Conversely, the more polar ones will be more related to the mobile phase, so they will elute earlier. This way, three different columns will be used in order to analyze the variations in the interaction of gangliosides with the column, which is reflected in the retention time, depending on the stationary phase used.

Depending on the polarity of the stationary phase, the interaction with certain ganglioside species will be greater or lesser, which is intended to be determined in this study.

In other studies carried out with the same objective of separating the different species of gangliosides, LC was also used with a high-sensitivity detection technology (ESI-MS), commonly used for lipids. The types of columns used can be either HPLC, which is the one used in this study, or UPLC (Ultra-High Performance Liquid Chromatography) can also be used, which due to its smaller packing particle size and therefore, Better packing allows for more effective separation.

Regarding the stationary phase most used for this type of technique, the reversed phase will be highlighted, since it allows a practically individual separation of the ganglioside molecules according to the length of their fatty acid chain and the number of unsaturations [19].

Although our study is going to be based on the analysis of gangliosides of the GM3 type, other experiments based on the study of other species of gangliosides have also been carried out. An example of this is found in the analysis of the glycosphingolipid GB3, described in the bibliography. [20]

Once the different types of gangliosides have been separated and eluted, they are detected in the mass spectrometer.

Mass spectrometer allows the structural characterization and specific quantification of gangliosides, performing a highly sensitive and selective analysis. Thereby, molecular masses of gangliosides are detected and measured with high accuracy, separating and distinguishing isomers, if any,

thus allowing the identification of the different species of gangliosides present in the analyzed sample. This technique has become a useful means in gangliosides characterization and identification [21].

The most suitable type of ionization mode for the GA analysis is negative ionization. This is due to the presence of the sialic acid on GG's structure, which will form negative ions. Acids are chemical compounds with a tendency to donate protons. Due to the negative charge sialic acids present and to the tendency of the carboxyl and amino groups of its structure to form negative ions, it makes it easier for the mass spectrometer to detect and quantify GG's with negative ionization. While it is true gangliosides also contain amino groups, where protons could be attached, the presence of sialic acid is much more important when selecting the ionization mode.

Thus, in order to carry out the physical separation of our target compounds and their subsequent detection and identification, LC-MS technique will be used. This technique is probably the most widely used in the characterisation of molecules involved in metabolic processes due to its ability to separate and detect a wide range of molecules according to their polarity and concentration.

Each component enters the MS detector at a different time, according to its affinity with the stationary and the mobile phase in the chromatographic column. Since the sample of pure gangliosides initially introduced into the device is made up of a mixture of different ganglioside species, they will first be separated by liquid chromatography according to their chemical composition. The different components will be separated, since each one will present a different retention time according to its affinity with the chosen chromatographic column, so they will reach the spectrometer detector at different times, making it easier to identify their peaks.

Each component ionizes in the ion source, producing ions with a certain charge and different mass numbers. As each component has acquired a certain charge, they can be separated by applying a magnetic field and thus be quantified according to their abundance.

3. EXPERIMENTAL PART

3.1 Materials and chemicals

In relation to the materials used for the experiment, we will highlight the column to perform the HPLC chromatography , as well as the mass spectrometer.

Thus, a stationary phase composed of hydrophilic materials, which are capable of providing strong interactions with polar analytes, is required for the column. The choice of the stationary phase to be used in HPLC depends mainly on the nature of the compounds to be separated. Although the most commonly used stationary phase in this technique is silica, it is extremely polar and therefore not the most suitable for the separation of gangliosides as it would also retain the polar groups. This is because, although gangliosides have an amphipathic character, a lipidic and hydrophobic part in addition to their polar character given by the hydroxyl and carbohydrate groups in their structure, they are considered polar in nature, since the hydrophilic part tends to predominate in the structure as a whole. However, the hydrophobic or hydrophilic character of each ganglioside molecule will depend on its structure, the number of unsaturations present, the length of the chain of fatty acids, the number of hydrophilic groups present, and the length of the chain.

As we intend to separate different ganglioside species within the GM3 class and therefore with different polarities, silica will not be used as a stationary phase. This phase tends to retain polar compounds for a longer period of time, resulting in longer retention times or inadequate separation in the case of polar compounds. In order to avoid this and to ensure a proper separation of all gangliosides, other hydrophobic or less hydrophilic stationary phases than silica, e.g. C18, will be used. This column is mainly composed of silica (SiO_2) but has been modified with hydrophobic groups: long carbon chains have been added to the SiO_2 molecule in order to ensure a correct separation of the gangliosides.

For this reason, the stationary phases to be used for the quantification and classification of the GG will be silica-derived phases, but modified with hydrophobic groups that allow for the correct separation of the gangliosides.

In our case, we will use three different types of columns: Biozen Oligo C18, biphenyl and F5.

The C18 column, for example, has the following structure C18H37, which gives it hydrophobic properties. From this carbon groups interact with the non-polar organic compounds in the sample, allowing them to be separated according to their differences in affinity for the stationary phase. The more hydrophobic compounds tend to be retained more on the C18 column, while the more polar compounds elute more rapidly.

However, the three phases do not have the same degree of hydrophobicity, so different retention and elution times will be obtained in each column for the same species of ganglioside.

The same happens with the biphenyl and F5 columns, which also interact selectively with the molecules, depending on the hydrophobic interactions that occur with the molecules to be analyzed.

In turn, sample vials made of inert materials are required, which contain the samples before they are injected into the chromatogram.

Regarding the solvents used, the use of two mobile phases will be highlighted in the first place. These consist of mixtures of water with some polar organic solvent: Said proportion of water: organic solvent can be adjusted throughout the chromatography.

- Mobile phase A: 80% methanol in water with 5mM ammonium formate
- Mobile phase B: 99% methanol in water with 5mM ammonium formate

On the other hand, it is worth noting the use of Buffer or salts such as ammonium formate both to improve the efficiency of ionization in the MS.

3.2 Sample preparation

The standard used is GM3 bovine at a concentration of 1 mg/mL, which was dissolved in a 2vpv (20 : 80) methanol water solution.

3.3 Techniques used for the extraction of gangliosides in biological samples.

Specifically, the study is going to be focused on the identification and classification of different subtypes of gangliosides within the GM3 category, so gangliosides which are going to be analyzed in the mass spectrometer are different subclasses of GM3 which vary on the length of the fatty acid and the sphingoid's length, on the number of double bonds and hydroxyl groups and on the composition of the oligosaccharide part. This way, the ceramide moiety can be attached to different sugars, among which it is worth mentioning glucose (Glc) or galactose (Gal), which are neutral sugar which can be attached, in its turn, to other neutral sugars such as N-acetylglucosamine (GlcNAc) or N-acetylgalactosamine (GlcNAc), or ionized/ charged groups, which are made up of the previously mentioned sugars and sulfur. Each subclass is expected to present a different retention time due to its ceramide portion's affinity with the stationary phase.

Table 1. Structures of the molecules that make up the chemical structure of gangliosides.

Name	Structure	Examples
Fatty acid	$CH_3(CH_2)_nCOOH$	Oleic acid Linoleic acid

Sphingosine	C18H37NO2	Ceramide Ganglioside GM3
Oligosaccharide	Between two and ten monosacharides	
Sialic acid	N-acetilneuraminic	

Therefore, the experiment was performed with the use of three different columns, which all had the same physical parameters such as pH, dimensions, pore size and only differed in the chemistry of the stationary phase, what is going to determine the differences in retention time. The chromatographic conditions were the same for all the analysis, e.g. flow rate, mobile phase composition, dimension of the column such as length, diametre, particle size

. The elution of gangliosides selected as the object of the study will be analyzed in: Biozen Oligo C18, biphenyl and F5.

In regards to these conditions, it should be highlighted that the chromatographic system used for this study was a system provided by Agilent Technologies. Two mobile phases were used:

- Mobile phase A: 80% methanol in water with 5mM ammonium formate
- Mobile phase B: 99% methanol in water with 5mM ammonium formate

The elution gradient program was as follows, initially starts with 100% of mobile phase A and 0% of mobile phase B. Gradually decrease the

concentration of A and increase the concentration of B until 0% of A and 100% of B. Once all or most of the ganglioside species had been eluted, which took about 25 minutes, it was returned to the initial proportions in which the mobile phase was composed of 100% A and a process was started to equilibrate the column before it was used again, which took about 10 minutes. Temperature of the column was 60°C.

The columns had the following characteristics 1.7 um 2.1 x 100 mm and was used at a temperature of 60ºC, temperature which will be increasing as the temperature gradient technique will be used, at 0.400 mL flow rate. Samples were kept at 7ºC during the analysis and the injection was standard and it´s volume was 1.00 uL. As for the mass spectrometer, the operating mode used for the analysis is the scan mode with a scan rate of 1.5 spectra/sec. This configuration allows the MS to scan, detect and register a wide range of ions present in the initial sample, according to their mass-to-charge ratio. We will work in SCAN mode, in mass range 400-3200 m/z, within which the MS will register the ions that are in that range.

Thus, the fragmenter will work with a voltage of 120V.

As for the nebulizer, this is a device used to volatilize the liquid sample in a kind of mist in the form of small droplets in the electrospray ionization technique, which is the technique used in the MS in our analysis. In this way, it is responsible for removing the liquid solvent from the introduced sample to obtain a uniform distribution of charged ions and in the gaseous state. For this, it consists of a gaseous current which comes into contact with the sample that is being pumped to the spectrometer at a pressure of 35 psig.

3.4. Gangliosides identification

After being analyzed in the MS, several GM3-type ganglioside species were detected, which each manifest themselves as a peak, according to their respective retention time, in the total ion chromatogram.

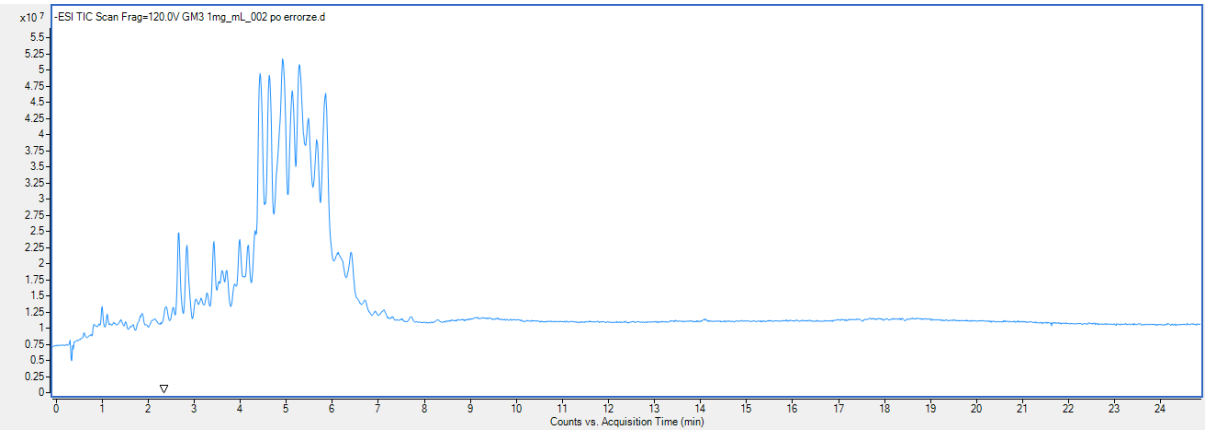

Figure 3. Total Ion Chromatogram of the peaks of the different species of gangliosides GM3 present in the sample.

Once each of the species has been differentiated, the type is determined by checking that their retention times coincide with those identified from the samples. However, said separation of the GGs can be hindered by the presence of phospholipids that could not be separated correctly in previous stages.

The results obtained after analysis, in each of the columns, are as follows:

Table 2. Gangliosides retention times in Biozen Oligo C18 column.

Name	m/z	Retention time (min)
GM3(d30:1)	1095.6422	2.002

GM3(d32:1)	1125.6841	5.758
GM3(d32:1)	1123.6722	5.156
GM3(d33:1)	1137.6922	6.01
GM3(d34:2)	1149.6922	5.705
GM3(d34:1)	1151.7022	5.705
GM3(d34:0)	1153.7222	6.855
GM3(d35:1)	1165.7159	7.755
GM3(d36:2)	1177.7222	7.555
GM3(d36:1)	1179.7322	8.66
GM3(d36:0)	1181.7522	9.33
GM3(d37:1)	1193.7522	9.615
GM3(d38:2)	1205.7522	9.31
GM3(d38:1)	1207.7722	10.413
GM3(d38:0)	1209.7822	11.00
GM3(d39:1)	1221.7822	11.22
GM3(d40:2)	1233.7822	10.659
GM3(d40:1)	1235.8022	11.94

GM3(d40:0)	1237.8022	11.94
GM3(d41:2)	1247.7925	10.81
GM3(d41:2) iso 1	1247.7925	10.61
GM3(d41:1) iso 2	1249.8122	11.91
GM3(d42:3)	1259.8022	10.35
GM3(d42:2)	1261.8122	11.36
GM3(d42:1)	1263.8322	12.68
GM3(d43:2) iso 1	1275.8231	12.11
GM3(d43:2) iso 2	1275.8231	12.12
GM3(d44:1)	1291.8551	9.23

Table 3. Gangliosides retention times in biphenyl column.

Name	m/z	Retention time (min)
GM3(d30:1)	1095.6422	1.08
GM3(d32:1)	1125.6841	2.2
GM3(d32:1)	1123.6722	2.065

GM3(d33:1)	1137.6922	2.34
GM3(d34:2)	1149.6922	2.43
GM3(d34:1)	1151.7022	2.65
GM3(d34:0)	1153.7222	2.84
GM3(d35:1)	1165.7159	3.03
GM3(d36:2)	1177.7222	3.15
GM3(d36:1)	1179.7322	3.43
GM3(d36:0)	1181.7522	3.43
GM3(d37:1)	1193.7522	3.97
GM3(d38:2)	1205.7522	4.09
GM3(d38:1)	1207.7722	4.43
GM3(d38:0)	1209.7822	4.63
GM3(d39:1)	1221.7822	4.93
GM3(d40:2)	1233.7822	4.87
GM3(d40:1)	1235.8022	5.29
GM3(d40:0)	1237.8022	5.29
GM3(d41:2)	1247.7925	5.23

GM3(d41:2) iso 1	1247.7925	5.23
GM3(d41:1) iso 2	1249.8122	5.42
GM3(d42:3)	1259.8022	5.85
GM3(d42:2)	1261.8122	5.78
GM3(d42:1)	1263.8322	5.79
GM3(d43:2) iso 1	1275.8231	6.31
GM3(d43:2) iso 2	1275.8231	6.34
GM3(d44:1)	1291.8551	5.78

Table 4. Gangliosides retention times in F5 column.

Name	m/z	Retention time (min)
GM3(d30:1)	1095.6422	9.22
GM3(d32:1)	1125.6841	1.77
GM3(d32:1)	1123.6722	1.63
GM3(d33:1)	1137.6922	1.08
GM3(d34:2)	1149.6922	1.87

GM3(d34:1)	1151.7022	2.14
GM3(d34:0)	1153.7222	2.34
GM3(d35:1)	1165.7159	10.34
GM3(d36:2)	1177.7222	2.36
GM3(d36:1)	1179.7322	2.83
GM3(d36:0)	1181.7522	2.83
GM3(d37:1)	1193.7522	3.35
GM3(d38:2)	1205.7522	3.14
GM3(d38:1)	1207.7722	3.8
GM3(d38:0)	1209.7822	4.07
GM3(d39:1)	1221.7822	4.31
GM3(d40:2)	1233.7822	3.85
GM3(d40:1)	1235.8022	4.66
GM3(d40:0)	1237.8022	4.67
GM3(d41:2)	1247.7925	4.2
GM3(d41:2) iso 1	1247.7925	4.4
GM3(d41:1) iso 2	1249.8122	5.23

GM3(d42:3)	1259.8022	4.62
GM3(d42:2)	1261.8122	4.72
GM3(d42:1)	1263.8322	5.8
GM3(d43:2) iso 1	1275.8231	3.79
GM3(d43:2) iso 2	1275.8231	5.24
GM3(d44:1)	1291.8551	3.98

The gangliosides that will be chosen for analysis are the following:

- GM3 (32d:0)
- GM3 (34d:0)
- GM3 (36d:0)
- GM3 (36d:1)
- GM3 (36d:2)
- GM3 (38d:0)

5. Results and discussion.

As mentioned above, gangliosides are molecules found in large quantities in the cells of the nervous system, as well as, to a lesser extent, in extraneural tissues. A good way to extract these gangliosides is by performing the consequent extraction and separation techniques on human milk, in order to isolate the gangliosides from the rest of the substances that surround it. However, as indicated above, the samples used in the experiment are not from human milk, but from bovine milk, since the concentration of gangliosides present in human milk is very low, which makes their extraction difficult.

Therefore, samples of purified bovine GM3 standard are injected into the LC-MS. RP is chromatographic mode through which it is possible to separate the hydrophobic compounds that make up a sample, such as gangliosides, for subsequent analysis. To carry out this separation, three different chromatographic columns will be used in which the hydrophobic compounds will have a greater interaction with the stationary phase, which is apolar and therefore hydrophobic, unlike the hydrophilic compounds which, having little affinity with the stationary phase, elute rapidly. For elution, the mobile phase used is a mixture of water and methanol (20:80).

Once the gangliosides have been separated according to their subspecies by reverse-phase chromatography, they are ready to be introduced into the mass spectrometer for analysis. This device will determine the structure and composition of the individual gangliosides.

In this way, 28 different subspecies of gangliosides were detected, which differed in ceramide moiety and structural issues such as the number of double bonds. Thus, each ganglioside is differentiated by two numerical digits "x" and "y" (dx:y), which distinguish it from the rest of the species. The first number refers to the length of the fatty acid chain attached to the polar part of the molecule. In other words, the number of carbons in the fatty acid chain. The second number indicates the number of unsaturated bonds, double bonds, present in the fatty acid chain. For this reason, the retention times for each type of ganglioside, according to the structure of its fatty acid chain, have been

analyzed in three different columns, in order to determine the influence of its interaction with the stationary phase on the retention time.

Thus, the three columns on which the analysis will be carried out have the same chromatographic conditions and differ only in the chemistry of the stationary phase. Thus, their interaction, and thus their retention time, will vary according to the hydrophobic or hydrophilic character of the column used. The three columns used being as follows:

- Biozen Oligo C18 Column
- Fluoropentyl (F5) Column
- Biphenyl Column

As can be seen, the retention times are very different from one column to another, so, in order to facilitate their analysis and consequent comparison, it has been decided to select three gangliosides with a chain of fatty acids of determined lengths (d32, d34, d36) and their respective variations with respect to unsaturations.

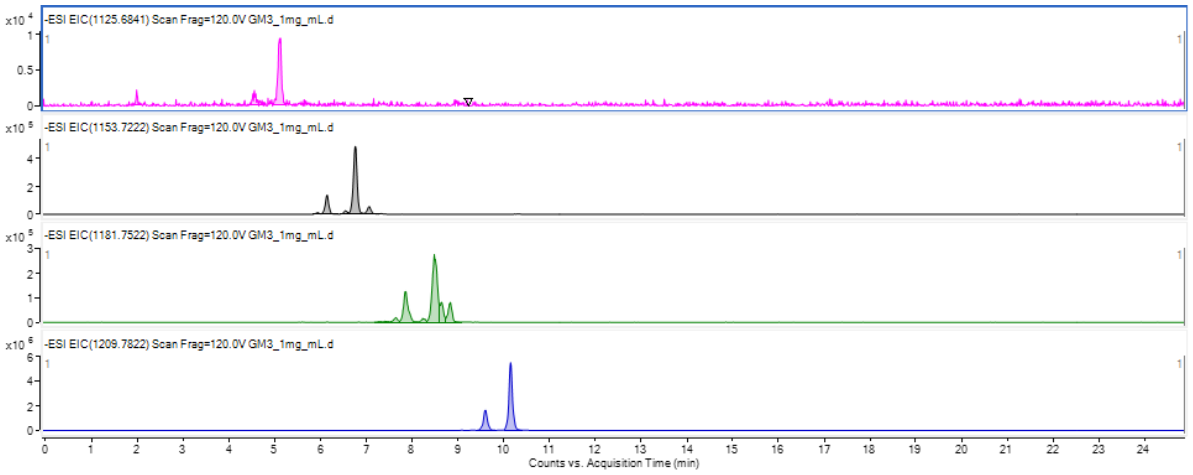

Figure 4. Extracted ion chromatograms of GM3 species GM3(32d:0), GM3(34d:0), GM3(36d:0), GM3(38d:0) differing in fatty acid chain lengths separated on a Biozen Oligo C18 column.

Table 5. Gangliosides retention times of selected species with different fatty acid´s length chain in Biozen Oligo C18 column.

Name	m/z	Retention time (min)
GM3(32d:0)	1125.6841	5.11
GM3(34d:0)	1153.7222	6.77
GM3(36d:0)	1181.7522	8.48
GM3(38d:0)	1209.7822	10.15

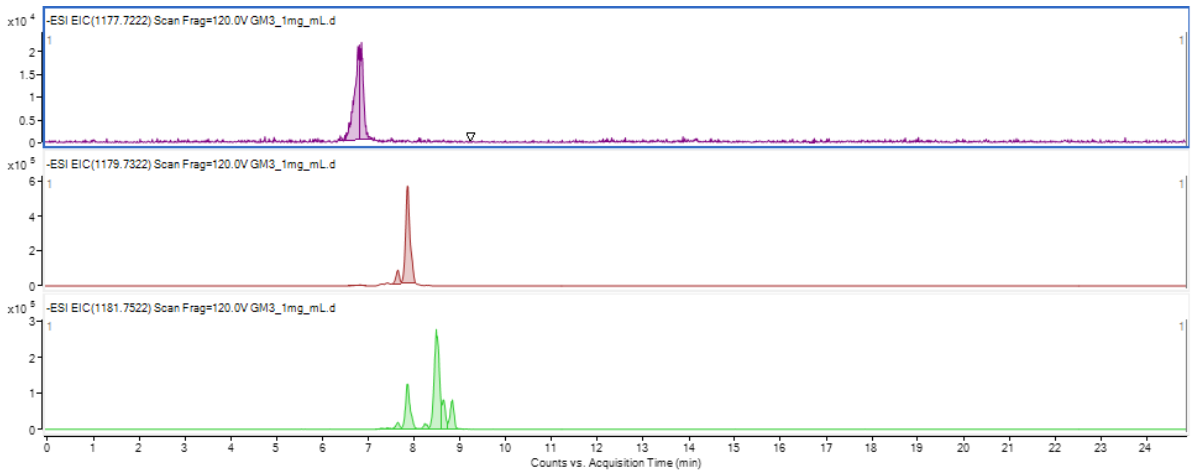

Figure 5. Extracted ion chromatograms of GM3 species GM3(36d:0), GM3(36d:1), GM3(36d:2) differing in numbers of unsaturated bonds on an Biozen Oligo C18 column.

Table 6. Gangliosides retention times of selected species with different number of unsaturated bonds in Biozen Oligo C18 column.

Name	m/z	Retention time (min)
GM3(36d:0)	1181.7522	8.48
GM3(36d:1)	1179.7322	7.86
GM3(36d:2)	1177.7222	6.80

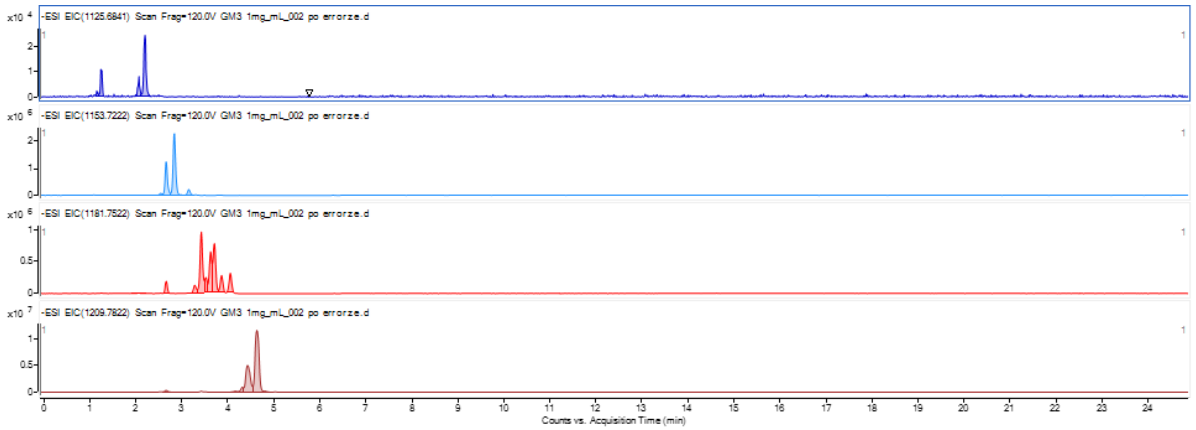

Figure 6. Extracted ion chromatograms of GM3 species GM3(32d:0), GM3(34d:0), GM3(36d:0), GM3(38d:0) differing in fatty acid chain lengths separated on a biphenyl column.

Table 7. Gangliosides retention times of selected species with different fatty acid´s length chain in biphenyl column.

Name	m/z	Retention time (min)

GM3(32d:0)	1125.6841	2.2
GM3(34d:0)	1153.7222	2.84
GM3(36d:0)	1181.7522	3.13
GM3(38d:0)	1209.7822	4.63

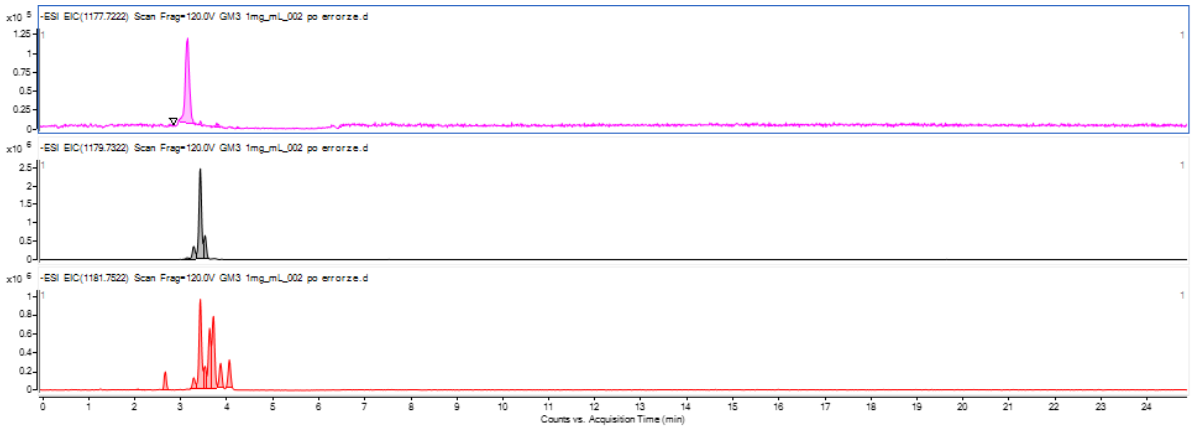

Figure 7. Extracted ion chromatograms of GM3 species GM3(32d:0), GM3(34d:0), GM3(36d:0), GM3(38d:0) differing in fatty acid chain lengths separated on an biphenyl column.

Table 8. Gangliosides retention times of selected species with different number of unsaturated bonds in biphenyl column.

Name	m/z	Retention time (min)

GM3(36d:0)	1181.7522	3.43
GM3(36d:1)	1179.7322	3.43
GM3(36d:2)	1177.7222	3.13

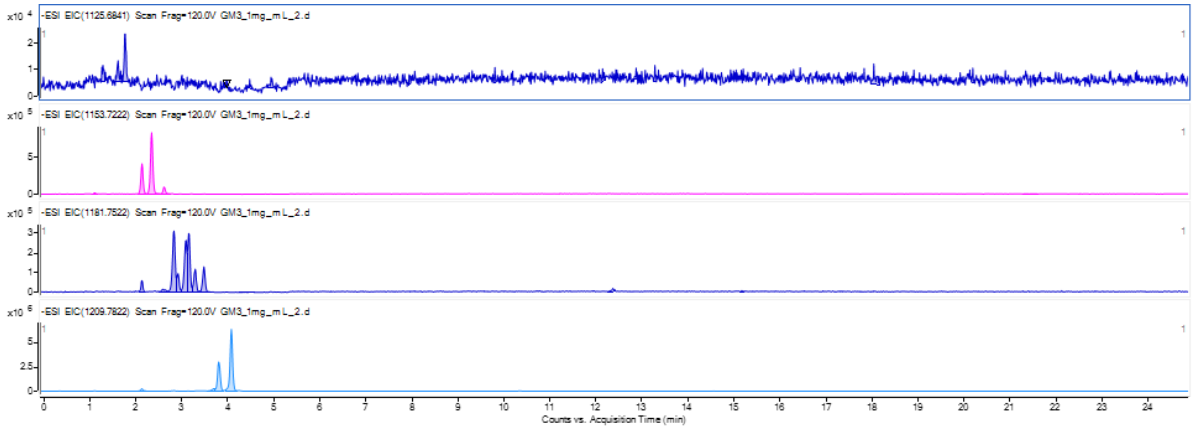

Figure 8. Extracted ion chromatograms of GM3 species GM3(32d:0), GM3(34d:0), GM3(36d:0), GM3(38d:0) differing in fatty acid chain lengths separated on a F5 column.

Table 9. Gangliosides retention times of selected species with different fatty acid´s length chain in F5 column.

Name	m/z	Retention time (min)
GM3(32d:0)	1125.6841	1.77
GM3(34d:0)	1153.7222	2.34
GM3(36d:0)	1181.7522	2.83

GM3(38d:0)	1209.7822	4.07

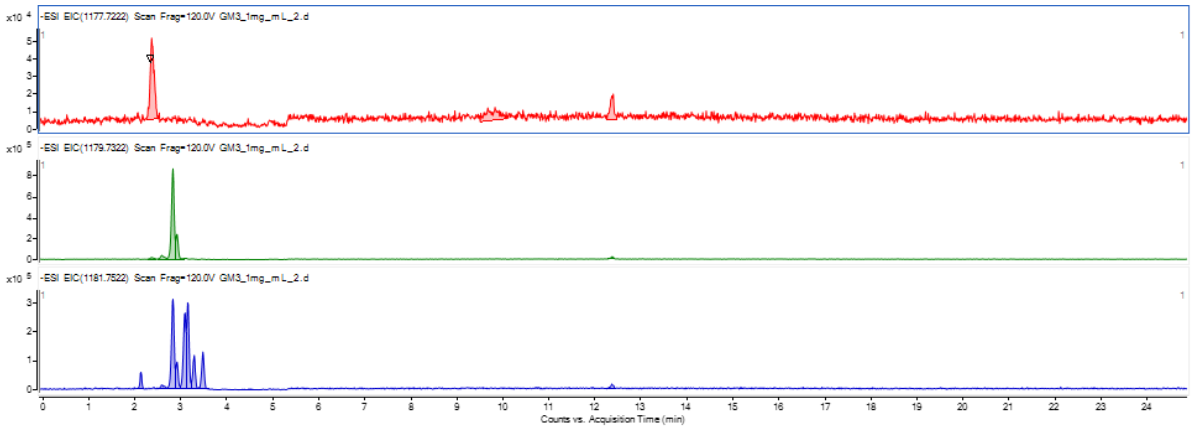

Figure 9. Extracted ion chromatograms of GM3 species GM3(36d:0), GM3(36d:1), GM3(36d:2) differing in numbers of unsaturated bonds on an F5 column.

Table 10. Gangliosides retention times of selected species with different number of unsaturated bonds in F5 column.

Name	m/z	Retention time (min)
GM3(36d:0)	1181.7522	2.83
GM3(36d:1)	1179.7322	2.83
GM3(36d:2)	1177.7222	2.36

Table 11. Comparison of gangliosides retention times of selected species with different fatty acid´s length chain in the three columns selected.

	32d:0	34d:0	36d:0	38d:0
Biozen Oligo C18	5,11	6,77	8,48	10,15
BIPHENYL	2,2	2,84	3,43	4,63
F5	1,77	2,34	2,83	4,07

Table 12. Comparison of gangliosides retention times of selected species with different number of unsaturated bonds in the three columns selected.

	36d:0	36d:1	36d:2
Biozen Oligo C18	8,48	7,86	6,8

BIPHENYL	3,43	3,43	3,13
F5	2,83	2,83	2,36

First, the retention times of each of the three columns will be compared, looking at the influence of the length of the fatty acid chain. Thus, gangliosides with chains of 32, 34, 36 and 38 carbons were compared.

As previously mentioned, fatty acids have a hydrophobic and a hydrophilic portion. Thus, as the number of carbons in the chain increases, the hydrophobic portion becomes more predominant over the hydrophilic portion, in other words, the polarity of the molecule decreases. The longer the retention time of the ganglioside molecule, the greater its affinity for the stationary phase, which has an apolar character. This is why, as was to be expected, in the three columns, the greater the number of carbons that make up the carbon chain, the less polarity the molecule presents and therefore its interaction with the apolar stationary phase will be much greater, being retained in it for longer and therefore presenting a longer retention time in the column.

Thus, in all three cases, the ganglioside species that crosses the column and reaches the detector in the shortest time is the one with 32 carbons in the chain, as it is the one that separates more easily from the stationary phase, moving through the column more quickly. On the other hand, it is not only the number of carbons, and therefore the polarity of the molecule, that influences the level of retention, but the molecule size is also a factor to be taken into account, which goes hand in hand with polarity. The smaller the molecule size, the easier it is for the molecule to pass through the column. Therefore, the lower the polarity, which implies a higher affinity for the hydrophobic stationary phase, and the higher the lipophilicity, the longer the ganglioside will be retained on the column.

A clear example of this can be found in the tables above, where in all three cases the ganglioside molecule with the longest retention time is the 38-carbon molecule, and the least is the 32-carbon molecule, with the 34-carbon and 36-carbon species in between, in increasing order of retention. Once the influence of the fatty acid chain length on the interaction with the column has been determined, the influence of the type of column on the same type of ganglioside will be analyzed.

The main factor affecting the variations in the time taken for the same ganglioside to reach the detector depending on the type of column used is lipophilicity. As can be seen, each molecule has a different retention time in each of the three chromatographic columns, which depends on the different selectivity of each of them, depending on the chemical composition of the stationary phase. A longer retention time implies a greater interaction of the molecule with the stationary phase. This is why, taking into account the times obtained, it can be concluded that the first column in which the experiment was carried out, Biozen Oligo C18, has a higher hydrophobicity. In the 4 examples of molecules taken for the analysis, longer retention times were obtained when using the Biozen Oligo C18 column, followed by the biphenyl and, finally, of F5. This last column is the one with the shortest times, so it can be said that it is the less hydrophobic of all and, therefore, the one that has the least interaction with the ganglioside molecules, since these cross the column more easily than in the other two previous cases, managing to be identified by the detector in the shortest time out of the three. That is why, the ganglioside that remains in the column for the longest time is the one with the longest carbon chain length in the column with the lowest polarity, Biozen Oligo C18, and the ganglioside that remains the least, the one with the lowest molecular weight and, therefore, , fewer carbons in the most polar column of the three, the F5, these being 10.15 and 1.77 minutes.

Regarding the influence of unsaturations in the fatty acid chain with respect to the mass spectrometer. This device is responsible for detecting and analyzing the compounds that have been previously separated in the chromatographic column according to the degree of ionization and

fragmentation of the compounds in the gas phase. The existence or not of double bonds in the chemical structure of the gangliosides affects the stability of the ions generated as well as their reactivity after fragmentation.

As for its influence on the retention time of the molecule in each of the three chromatographic columns, the polarity of the molecule is again a significant factor. The existence of double bonds in the ganglioside usually leads to lower retention times. This is because they tend to be more unsaturated and therefore less hydrophobic, what increases the polarity of the molecule, as a consequence of the negative charge generated by the pi electrons in the double bond. This decrease in lipophilicity results in a weaker interaction with the stationary phase and thus a shorter elution time.

On the other hand, specific interactions between the double bonds of the molecule and the hydrocarbon chains of the stationary phase have to be taken into account, thus increasing the retention time of the molecule in the column. Also, the existence of double bonds allows the possibility of hydrogen bridging or intermolecular interactions with the bonds in the stationary phase, which would also increase retention. Since the presence of the double bonds increase the polarity of the molecule, making the molecule less hydrophobic, the less double bonds the molecule has, the more likely it is to interact with the intermolecular forces of the hydrophobic stationary phase and thus the more it will be retained in the column. The ganglioside molecules that show the greatest interaction with the column are, in all three columns used: Biozen Oligo C18, biphenyl and F5 , those that do not have any double bonds in their structure. This means that the more apolar the ganglioside species is, the more affinity it has with the chosen stationary phases, which concludes the hydrophobic character of the three chosen columns. The fact that, when dealing with a hydrophobic stationary phase, a larger number of double bonds usually results in more polar molecules which, therefore, have a lower affinity and, with it, less interaction with the stationary phase. Therefore, those molecules with 1 or 2 double bonds will elute earlier than those without double bonds. In this way, ganglioside species with fewer double bonds in their structure will will have greater difficulty passing through the column as a

consequence of their higher affinity for the hydrophobic stationary phase, resulting in longer retention times for those molecules with fewer double bonds in their structure.

That is why it can be openly concluded that, for the same ganglioside, with the same structure in the fatty acid chain, the retention time varies depending on the column used. Since the chromatographic conditions of the three columns selected for analysis are the same and the only thing that varies between them is the composition of the stationary phase. Depending on its chemical composition, it will present a greater or lesser polarity, which will lead to a greater or lesser interaction with the ganglioside molecules. In this way, as the three columns present a predominantly nonpolar character, the species of gangliosides with a longer carbon chain and fewer double bonds are the ones that will remain retained longer in the column, since the nonpolar proportion will predominate over the nonpolar one. polar. Conversely, the fewer carbons the fatty acid chain has, the less weight the nonpolar part will have in the molecule, so there will be fewer interactions with the nonpolar stationary phase. The same happens with the double bonds, which provide the molecule with polarity. The fewer the number of double bonds, the lower the polarity and, therefore, the greater the affinity with the column in question.

After obtaining the values of the different retention times, it is concluded that the most polar column and that, therefore, it will have the least interaction with the nonpolar part of the gangliosides, is the F5 column, since the molecules are capable of cross it with hardly any difficulty and reach the detector in the shortest possible time. Thus, in all the analyzes it is the one with the shortest retention time and, therefore, the most polar of the three. On the contrary, the Biozen Oligo C18 column is the one with the longest retention times and, therefore, the highest affinity with the apolar part of the ganglioside molecules, concluding, therefore, that it is the most non-polar column of the three.

Regarding the course of the experiment and the way of proceeding in the laboratory, it should be noted that techniques and methods similar to those applied in fields of study with similar objectives have been used.

For example, a study based on the intact glycosphingolipidomic analysis of the cell membrane during differentiation, carried out by the scientist Maurice Wong, was also performed by HPLC-MS and aimed at determining the glycan composition and sequence, as well as variations in the ceramide portion. The main objective was the identification and quantification of different intact GSL species on the cell surface of human tissue.

As in our study, the different species, according to the differences in their ceramide structure, are based on the length of their lipid chain and the number of unsaturations. Thus, the various GSL species were identified based on their monoisotopic masses verifying their structures with MS data.

In the aforementioned study, a solution of 50 uL of methanol/water 1:1 was used to reconstruct the samples, which were subsequently analyzed with an Agilent 6520 Accurate Mass Q-TOF LC/MS equipped with a C18 chip with analytical column. and enrichment.

In turn, a binary gradient was used with a flow rate of 0.3 uL/min:

- Mobile phase A: 20 mM ammonium acetate and 0.1% acetic acid in water.

- Mobile phase B: 20 mM ammonium acetate and 0.1% acetic acid in 85:15 (v/v) methanol/isopropanol.

In this way, the equipment was programmed so that the elution percentage of solvent B was linearly increased from 80% to 100% over 36 minutes. On this occasion, unlike in our study, the mode used was positive.

Thus, in an experiment carried out under other conditions but with the same type of column, C18, the result is the same, with a shorter retention time for molecules with a more hydrophilic character, given the hydrophobic character

of the column. This is why molecules with shorter carbon chains and a greater number of unsaturations have the shortest retention time in the hydrophobic column. [13]

On the other hand, in a study carried out on the separation of samples using the HPLC technique, it is observed in an experiment carried out with the same sample, under the same temperature conditions and with the same mobile phase and gradient but different stationary phase.

Since the polarity of the HPLC column is determined by the nature of its stationary phase, the use of various stationary phases in an experiment under the same conditions will cause the observed variations in retention times to be due exclusively to affinity or interactions. hydrophobic between the sample components and the stationary phase. Thus, the phases used in the analysis were SB-C8, SB-CN, SB-Phenyl, Extend-C18, SB-C18.

Thus obtaining the shortest retention time in the SB-CN column. This is because the sample to be analyzed probably has a hydrophilic character, quite similar to the polar SB-CN column, presenting a retention time of 1.8 to 2.3 minutes. The polarity of said column is given by the presence of cyano groups, which makes it more suitable for the separation of more polar compounds.

On the contrary, other columns such as the extend C-18, which has octadecylsilane groups (C18) in its structure, which makes it less polar than the previous one and more suitable for the separation of more hydrophobic compounds. The retention time in this case for the same sample is longer, from 2.3 to 3.4, thus concluding that the sample analyzed has a greater affinity with the Extend-C18 column and, therefore, a more hydrophobic character. That is why, for better separation, a less polar column should be used, but not excessively so, in order to avoid excessively long retention times. [22]

CONCLUSIONS

It has therefore been possible to evaluate the retention and elution order of GM3 species on three different stationary phases.

In this way, it should be noted that these physical properties depend mainly on the polarity of the functional group and the molecular mass. Thus, the identification of different species of GM3 class gangliosides will be based on the differences in their structure, which are mainly determined by the fatty acid chain length and number of unsaturations.

Thus, it has been shown that, for the separation and quantification of GG, mainly polar substances, despite their amphipathic character, it will be convenient to use a hydrophobic column, so that the molecules of interest in the sample do not interact excessively with the column and, therefore, their retention times are not excessively high. As has been observed in the three columns used, the more hydrophobic the column used, the less interaction they will have with the polar part of the GG molecules and therefore their elution time will be longer. This has been observed when repeating the experiment with the same chromatographic conditions and with the same samples, varying only the stationary phase, observing how the retention time varied in each case, being longer in the case of the more hydrophobic column and shorter in the case of the more hydrophilic column.

Thus, taking into account the length of the carbon chain of the ceramide and its number of unsaturations, it has been concluded that the greater the number of double bonds, the greater the polarity of the molecule, due to the partial charges generated between the atoms that make up the bond because of the unequal distribution of electrons between them as a result of their different electronegativity. Therefore, those species with a greater number of unsaturations are more hydrophilic and therefore have a shorter elution time.

On the contrary, as the carbon chain is larger, the hydrophobic part of the molecule increases and, with it, its interaction with the column, giving rise to a longer retention time, which has been observed in the graphical results obtained from the HPLC system.

It can be concluded that both the physical structure of the molecules to be quantified as well as the chemical nature of the HPLC column used in this thesis.

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