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Application of lyso-glycosphingolipid screenings for detection of inborn errors

MASTER'S THESIS

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Supervisor

Priv.-Doz. Mag. Dr.rer.nat. Günter Fauler

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Affidavit

I declare that I have authored this thesis independently, that I have not used other than the declared sources/resources, and that I have explicitly indicated all material which has been quoted either literally or by content from the sources used. The text document uploaded to TUGRAZonline is identical to the present master‘s thesis.

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I. Acknowledgement

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II. Abstract

Background: Lysosomal storage diseases are caused by inherited deficiencies of genes, encoding for lysosomal enzymes. These defective lysosomal enzymes lead to an accumulation of different enzyme substrates in different biological fluids. These accumulating products are associated with the pathogenesis of lysosomal storage diseases. Furthermore it has been proposed to use them for the diagnosis of lysosomal storage diseases. Especially the accumulating *lyso*-sphingolipids have attracted significant interest over the last few years and were quoted as potential biomarkers. The following diseases and their associated *lyso*-sphingolipids were in focus of our interest, GM1-gangliosidosis, GM2-gangliosidoses (Tay-Sachs disease, Sandhoff disease, AB-variant of GM2-gangliosidosis), Fabry disease, Gaucher disease, Metachromatic leukodystrophy, Krabbe disease and Niemann-Pick disease. The aim was to develop a single LC-MS/MS method, covering the diseases mentioned above, by analysing the accumulating *lyso*-sphingolipids in plasma samples.

Methods: The plasma samples were prepared with solid-phase extraction for the analysis with liquid chromatography coupled with a Q-Exactive high resolution mass spectrometer (LC-MS/MS). Three different LC-MS/MS methods were developed for the analysis of the following *lyso*-sphingolipids: psychosine, glucosylsphingosine, *lyso*-Gb3, *lyso*-sphingomyelin, *lyso*-gangliosides (GM1, GM2, GM3) and *lyso*-sulfatide.

Results: With the developed LC-MS/MS methods it is possible to distinguish between patients with Gaucher disease and Krabbe disease, and it is also possible to quantify their accumulating substrates psychosine and glucosylsphingosine. Furthermore it is possible to quantify the accumulation products, *lyso*-Gb3 in patients with Fabry disease and *lyso*-sphingomyelin in patients with Niemann-Pick disease. For the *lyso*-gangliosides and *lyso*-sulfatide a LC-MS/MS method was developed, but it was not possible to quantify them, due to the lack of an adequate internal standard.

Conclusion: It is possible to detect and quantify *lyso*-sphingolipids with the developed LC-MS/MS methods.

III. Kurzzusammenfassung

Hintergrund: Lysosomale Speicherkrankheiten werden durch vererbte Defekte in Genen, die lysosomal Enzyme kodieren, verursacht. Durch diese defekten lysosomalen Enzyme kommt es zu einer Ansammlung von Enzymsubstraten in verschiedenen biologischen Flüssigkeiten. Diese akkumulierenden Produkte werden ebenfalls mit der Pathogenese von lysosomalen Speicherkrankheiten in Verbindung gebracht. Des Weiteren wurde gezeigt, dass sich diese akkumulierenden Produkte auch zur Diagnosestellung eignen könnten. Vor allem die *lyso*-Sphingolipide zogen großes Interesse auf sich und wurden als potentielle Biomarker eingestuft. Von besonderem Interesse waren hierbei die folgenden Krankheiten mit ihren zugehörigen *lyso*-Sphingolipide: GM1-Gangliosidose, GM2-Gangliosidose (Morbus Tay-Sachs, Morbus Sandhoff, GM2-Gangliosidose AB-Variante), Morbus Fabry, Morbus Gaucher, metachromatische Leukodystrophie, Morbus Krabbe und Morbus Niemann-Pick. Das Ziel war eine LC-MS/MS Methode zu entwickeln, mit der es möglich ist die akkumulierenden *lyso*-Sphingolipide, der oben erwähnten Krankheiten, in Plasmaproben zu messen.

Methoden: Die Plasmaproben wurden mittels Festphasenextraktion, für die Analyse mit Flüssigchromatographie gekoppelt mit Q-Exactive, einem hochauflösenden Massenspektrometer, vorbereitet. Es wurden drei verschiedene LC-MS/MS Methoden entwickelt um folgende *lyso*-Sphingolipide Psychosin, Glucosylsphingosin, *lyso*-Gb3, *lyso*-Sphingomyelin, *lyso*-Ganglioside (GM1, GM2, GM3) und *lyso*-Sulfatid zu analysieren.

Resultate: Mit den entwickelten LC-MS/MS Methoden ist es möglich Patienten mit Morbus Gaucher und Morbus Krabbe zu unterscheiden und auch die zugehörigen akkumulierenden Substanzen, Psychosin und Glucosylsphingosin, zu quantifizieren. Desweitern ist es möglich die akkumulierenden Substanzen, *lyso*-Gb3 in Morbus Fabry und *lyso*-Sphingomyelin in Morbus Niemann-Pick, zu quantifizieren. Für die *lyso*-Ganglioside und *lyso*-Sulfatid wurde eine LC-MS/MS Methode entwickelt, jedoch war es nicht möglich diese zu quantifizieren, da kein passender Interner Standard zur Verfügung stand.

Fazit: Es ist möglich die *lyso*-Sphingolipide mit den entwickelten Methoden zu detektieren und zu quantifizieren.

IV. Contents

1. Introduction

A variety of inborn lysosomal storage diseases are caused by an inherited defect of different genes. The following lysosomal storage diseases were of particular interest, GM1-gangliosidosis, GM2-gangliosidoses (Tay-Sachs disease, Sandhoff disease, AB-variant of GM2-gangliosidosis), Fabry disease, Gaucher disease, Metachromatic leukodystrophy, Krabbe disease and Niemann-Pick disease and are the results of inherited deficiencies in the genes encoding for lysosomal enzymes. These lysosomal storage diseases are classified as sphingolipidoses, according to their accumulation product the sphingolipids. Also the pathogenesis of lysosomal storage diseases is associated with the accumulation of glycosphingolipids in the metabolic pathway. Especially, *lyso*-sphingolipids have attracted significant interest over the last few years and were quoted, in different publications, as potential biomarkers for the identification and the monitoring of different lysosomal storage diseases.

The aim of the master thesis was to develop a LC-MS/MS screening method for the different lysosomal storage disorders like GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, Fabry disease, Gaucher disease, Krabbe disease, metachromatic leukodystrophy and Niemann-Pick disease. The idea to develop such a screening method was to limit the effort of analysis and to facilitate the diagnosis of lysosomal storage disease patients, because the separate laboratory analysis for each disease is quite laborious. The screening method, with LC-MS/MS, should be based on the analysis of the different *lyso*-sphingolipids in plasma, which are accumulated in patients suffering from a lysosomal storage disease. These accumulating *lyso*-sphingolipids are psychosine, glucosylsphingosine, *lyso*-Gb3, *lyso*-sphingomyelin, *lyso*-gangliosides (GM1, GM2, GM3) and *lyso*-sulfatide. The first intention was to develop a single LC-MS/MS method analysing all different accumulating *lyso*-sphingolipids within a single run. Also a single plasma sample preparation which is adequate for the analysis of all different accumulating *lyso*-sphingolipids, had to be developed. After some further reflections it was clear that it is not possible to cover all diseases within a single screening method, because the accumulating *lyso*-sphingolipids are quite diverse in retention and ionisation behaviour.

2. Theoretical background

2.1. The Lysosome

The lysosomes are organelles in eukaryotic cells which are membrane-enclosed and contain about 40 different hydrolytic enzymes. These hydrolytic enzymes degrade a wide range of biological macromolecules like nucleic acids (DNA, RNA), proteins, oligosaccharides, glycosaminoglycans and lipids into their initial building blocks. The hydrolytic enzymes are acid hydrolases like proteases, nucleases, lipases, phospholipases, phosphatases, glycosidases and sulfatases. These acid hydrolases require an acidic pH-value around pH 5, whereas in other compartments in the cell the pH is neutral. This is a protection for the cell against uncontrolled degradation. On one hand compartment separation and on the other hand the pH-value is protecting the cell. The digesting enzymes are active in the membrane-enclosed lysosomes where the internal acidic pH is maintained. The acidic pH in the lumen is maintained by the proton pump (*$H^+ATPase$*) located in the lysosomal membrane. The perimeter membrane of lysosomes has to be protected against degradation. This is achieved by the so called glycocalyx, consisting of glycoproteins which are highly N-glycosylated with polylactosamine units and a high cholesterol level.

The lysosome is one component of the endosomal-lysosomal system, which includes the early endosomes, late endosomes and the lysosomes. For degradation, the macromolecules have to be taken up into the endosomal-lysosomal system, this is achieved with endocytosis, autophagy, phagocytosis or direct transport. The macromolecules from extracellular compartments are taken up by endocytosis or phagocytoses. The endocytosis can be a receptor mediated process or is performed without receptors. The internalized macromolecules are shuttled through the endosomal compartment and are finally taken up by the lysosomes as intra-lysosomal membrane structures. Whereas, with autophagy, cytoplasm or whole organelles are shuttled via an autophagosome to the lysosome and get fused with it. With phagocytosis larger particles or microorganisms are engulfed and are shuttled in form of phagosomes to the lysosomes. The degradation of glycosphingolipids is described in *2.3.*, in detail. The lysosomal enzymes and activator proteins which are synthesised in the rough endoplasmic reticulum (ER) have to be delivered to the lysosomes. The first step is the translocation through the endoplasmic reticulum to the lumen of the ER. This is achieved through an N-terminal signalling sequence. In the lumen

of the ER the signal sequence is cleaved of and the lysosomal enzymes undergo a N-glycosylation. After that, they are shuttled to the Golgi network and there the lysosomal enzymes receive a mannose 6-phosphate (M6-P) marker. The M6-P marker on lysosomal enzymes is required to enter the lysosomes. The uptake of lysosomal enzymes into the lysosomes is achieved through the formed receptor-protein complex. After degradation of the macromolecules in the lysosomes, the small resulting building blocks leave the lysosomes via diffusion or with the help of specialized transporter. The building blocks released to the cytosol are further degrade or reutilized for the metabolic pathway.

Defects in the lysosomal enzymes or activator proteins cause lysosomal storage disorders. A defect in a single enzyme or activator protein leads to an accumulation of enzyme substrate. The lysosomal storage disorders are specified over their accumulation products. According to the accumulation product the following diseases mucopolysaccharidoses, mucolipidoses, sphingolipidoses, glycoprotein and glycogen storage disorder are classified (*1–4*).

2.2. Structure and function of sphingolipids and glycosphingolipids

The sphingoid base, especially the sphingosine shown in figure 1, builds the basic structure of glycosphingolipids and it is characterized by the length of the alkyl chain. These alkyl chains have lengths between 14-20 carbons. On position 1 and 3 a hydroxyl-group and on position 2 an amino-group is substituted. The backbone of the sphingoid base may vary in length, but in human the alkyl chains usually have a length of 18 carbons, but also 20 carbons in length are possible. The species with a backbone length of 20 carbons occur in brain gangliosides. The alkyl chain is commonly single unsaturated in human, whereas in plants and fungi double bonds at different positions can occur. Further hydroxylation of the alkyl chain is possible at position 4 in human.

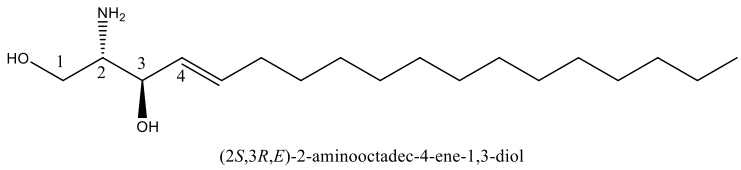

Figure 1: Structure of sphingosine (d18:1)

In general the sphingoid base is present in small amounts in the cell and usually is available as ceramide (Cer) shown in figure 2. In the formation of ceramide the sphingoid base is conjugated with and fatty acid at the amino group at position 2. The chain length of the fatty acids vary between 14-32 carbons and the fatty acids are usually saturated, but can have a single double bond or a hydroxyl group attached (*5*). Ceramides are important membrane building blocks and due to the saturation of the alkyl chains, the rigidity of the membrane is increased. Furthermore the transbilayer movement of molecules and the membrane curvature are influenced by ceramides. They also play an important role in cell signalling and cell growth regulation (*6*).

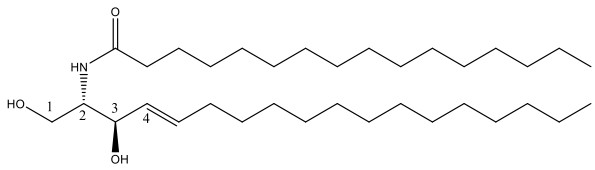

Figure 2: Structure of ceramide (Cer) (d18:1/16:0)

Furthermore different headgroups are linked via position 1 to the ceramides. The headgroups are differentiated by their polarity, polar or non-polar. An example for non-polar headgroups are fatty acids. Whereas phosphosphingolipids, neutral and neutral/acidic glycosphingolipids

belong to the ceramides with polar headgroups. The headgroups in phosphosphingolipids are linked via a phosphodiester bond to the ceramide. Examples for phosphosphingolipids are sphingomyelin and phosphoethanolamine. The neutral and neutral/acidic glycosphingolipids are divided into different subgroups which is shown in figure 3.

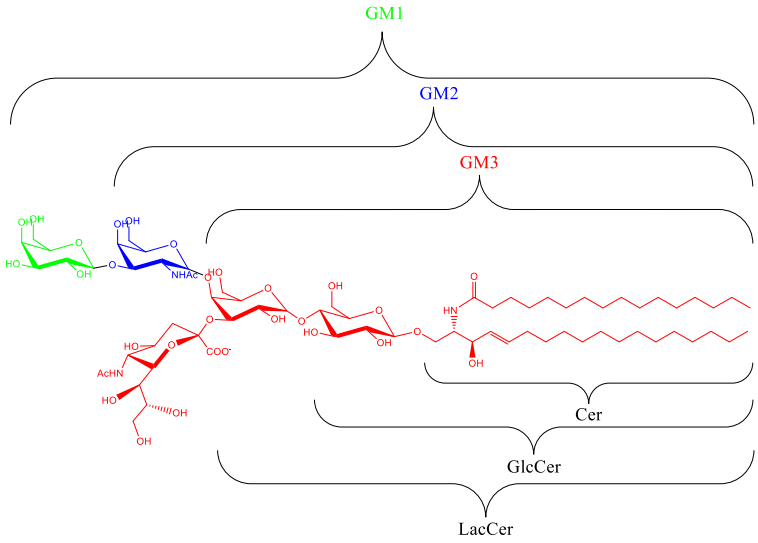

Figure 3: Scheme of neutral and neutral/acidic glycosphingolipids

The division starts with the first glycosidic β-linkage to ceramide at position 1, which is either glucose (glucosylceramide; GlcCer) or galactose (galactosylceramide; GalCer). Then the division is extended by additional substituents like carbohydrates or sulfate. The sulfatide is a GalCer derivative with sulfate linked to GalCer (3-sulfo-Galβ1Cer). The LacCer (lactosylceramide) is the result of a galactose addition to GlcCer (Galβ1-4Glcβ1Cer). The characterisation described previous is visualised in figure 3. The LacCer is an intermediate substance, which is necessary for the biosynthesis of globosides and gangliosides. The globoside, globotriaosylceramide (Gb3), is formed with further addition of galactose to LacCer (Galα1-4Galβ1-4Glcβ1Cer). Whereas the gangliosides contain neutral glycosphingolipids plus an acidic group the N-acetylneuraminic acid, also called sialic acid. There are two different guidelines for systematic naming of ganglioside, the IUPAC-IUB and the "Svennerholm" nomenclature. The "Svennerholm" nomenclature starts with G for gangliosides and is followed by the number of N-acetylneuraminic acid residues, thereby the M refers to the prefix "mono". So the full name of GM1 is monosialoganglioside GM1. The number in "Svennerholm" nomenclature refers to the gangliosides relative position in thin-layer chromatography (GM3 >

GM2 > GM1). The IUPAC nomenclature for GM3 is Neu5Acα2-3Galβ1-4Glcβ1Cer, for GM2 Neu5Acα2-3(GalNAcβ1-4)Galβ1-4Glcβ1Cer and Neu5Acα2-3(Galβ1-3GalNAcβ1-4)Galβ1-4Glcβ1Cer refers to GM1. Whereas Neu5Ac refers to N-acetylneuraminic acid and GalNAc to N-acetylgalactosamine (*5, 6*) The structure of GM3, GM2 and GM1is also shown in figure 3.

The glycosphingolipids together with cholesterol, sphingolipids and glycerophospholipids are the major components of eukaryotic cell surface membranes and have a variety of different functions (*3*). The membranes containing complex sphingolipids have unique surface characteristics and fluidity, which refers to cholesterol and sphingolipid rafts. Furthermore glycosphingolipids on cell surfaces are interacting with proteins on the same and neighbouring cell, extracellular matrix and also with bacteria or viruses. These interactions are named "glycosynapse". In addition sphingolipids are involved in membrane dynamics and also in cell regulation via membrane trafficking and metabolic interconversion (*6*).

As mentioned glycosphingolipids have a lot of different functions in the following a few examples are mentioned. GlcCer, for example, functions as a precursor for skin ceramides. Furthermore it is required for cell proliferation and survival, and also for intracellular membrane transport. The major components of myelin are GalCer and sulfatide. It has also been proposed that GalCer and sulfatide interact with each other via trans-carbohydrate-carbohydrate interactions. Moreover the sulfatide is supposed to be involved in myelin formation and maintenance, and neuronal cell differentiation. The LacCer is reported to function in cell signalling pathways which are involved in cell proliferation, migration, adhesion, angiogenesis, inflammation and phagocytosis. The ganglioside GM3 plays an important role in regulation of cell proliferation. The regulation is achieved through interactions via glycan-glycan binding on the EGF (epithelial growth factor) receptor and the result is stimulation of EGF. The functions of GM1 are similar to GM3, it also inhibits growth stimulation, but it acts on PDGF (platelet derived growth factor) instead of EGF. The gangliosides do not only contribute to membrane properties like charge, morphology, fluidity and polarity, they also modulate protein functions via interaction of surface proteins. The globoside Gb3 achieved attention, because of its accumulation in Fabry patients. Furthermore Gb3 is bound by verotoxins, Shiga toxin and the HIV adhesion gp120. The behaviour of these proteins in cell is significantly affected by the lipid backbones of Gb3 and probably important for the final pathogenic outcome (*6*).

2.3. Biosynthesis and degradation of sphingolipids and glycosphingolipids

Glycosphingolipid biosynthesis

The *de novo* glycosphingolipid biosynthesis starts with the formation of the hydrophobic ceramides at the cytoplasmic face of the ER. For glycosylation the ceramides are transported via vesicular transport or with the ceramide transfer protein (CERT) to the cytoplasmic face of Golgi apparatus. There the first glycosylation to GlcCer takes place. As described in *2.2. Structure and function of sphingolipids and glycosphingolipids*, the glucose is linked via β-glycosidic linkage to ceramide at position 1. The further addition of carbohydrates takes place in the luminal face of Golgi. Therefor GlcCer has to be translocated from the cytoplasmic face to the luminal face of Golgi. The translocation is probably achieved through the transport back to the ER, there it translocates to the luminal surface of the membrane and from there GlcCer can shuttle to the luminal face of the Golgi. After translocation GlcCer is converted to LacCer via lactosylceramide synthase. The further elongation of LacCer with carbohydrates is catalysed by the membrane bound glycosyltransferase on the luminal side of Golgi. The gangliosides are formed at the luminal surface of Golgi and the *trans*-Golgi network (TGN) membrane. There the sialic acid groups are linked to LacCer and the ganglioside precursors, mono-, di- and tri-sialoganglioside result. These are then converted to complex gangliosides via membrane bound glycosyltransferases. The products of glycosphingolipid biosynthesis are transported to the plasma membrane by vesicular transport (*3*, *7*).

Glycosphingolipid degradation

The degradation of glycosphingolipids by the lysosomes is an essentially process for the cell, because the resulting building blocks are nutrients for salvage and recycling pathway, but they also function as fuel for the energy metabolism (*3*).

First of all the glycosphingolipids located on extracellular surface have to reach the lysosomal compartment, this is achieved with endocytosis and the corresponding endocytic pathway. They reach the lysosomal compartment either as intra-endosomal vesicles or intra-endosomal membranes. In the first step of endocytosis the extracellular glycosphingolipids are internalised via non- or clathrin-coated pits and are fused with the early endosome. At the early endosome the pits are invaginated and bud into the lumen of the early endosome. The early endosomes mature to the late endosomes and the extracellular components, targeted for degradation,

undergo different sorting processes before they are delivered as intra-lysosomal vesicle to the lysosomal compartment. Before they are delivered as intra-lysosomal vesicle, the intra-lysosomal membrane undergo lipid-sorting. These lipid-sorting processes contain a decrease of cholesterol content and an increase of the negatively charged bis(monoacylglycero)phosphate (BMP). The intra-lysosomal membranes are enriched in BMP, whereas BMP is nearly absent in the perimeter membrane. The perimeter membranes are enriched in glycoproteins, which are N-glycosylated with polylactosamine units, and in cholesterol. The cholesterol concentration in inner lysosomal membranes is regulated by sphingomyelin and ceramide. The sphingomyelin is degraded during the endocytic pathway by sphingomyelinase to ceramide. The ceramide is then degraded by ceramidase, whereas the cholesterol is not degraded by the lysosomes. It is shuttled out of the lysosomes, which is achieved by cholesterol transporters (NPC1/NPC2). The lipid-sorting process is performed to provide an optimal lipid composition of the intra-lysosomal membrane, for intra-lysosomal vesicle degradation. Then glycosphingolipids are degraded by a stepwise release of carbohydrate units linked to the headgroups, performed by the lysosomal enzymes at the acidic pH optimum. The lysosomal enzymes are water soluble, whereas their substrates, the glycosphingolipids, are anchored in the intra-lysosomal membranes. That means that the hydrophilic lysosomal enzymes are not able to attack the amphiphilic glycosphingolipids. To handle this phase problem, small water-soluble lipid-transfer and lipid-binding glycoproteins, the sphingolipid activator proteins (SAPs or saposines) are necessary. The sphingolipid activator proteins include five glycoproteins, the Sap A-D and the GM2 activator protein (GM2-AP), which are encoded by two genes. The glycosphingolipids, substrates of the lysosomal enzymes, are embedded in the intra-endosomal or intra-lysosomal membrane. The SAPs facilitate the accessibility of the substrate for the degrading enzymes (*3, 7, 8*).

In figure 4 the sphingolipid degradation and there corresponding degrading enzymes and SAPs, in red and the corresponding lysosomal storage disorder, in blue, are schematically shown.

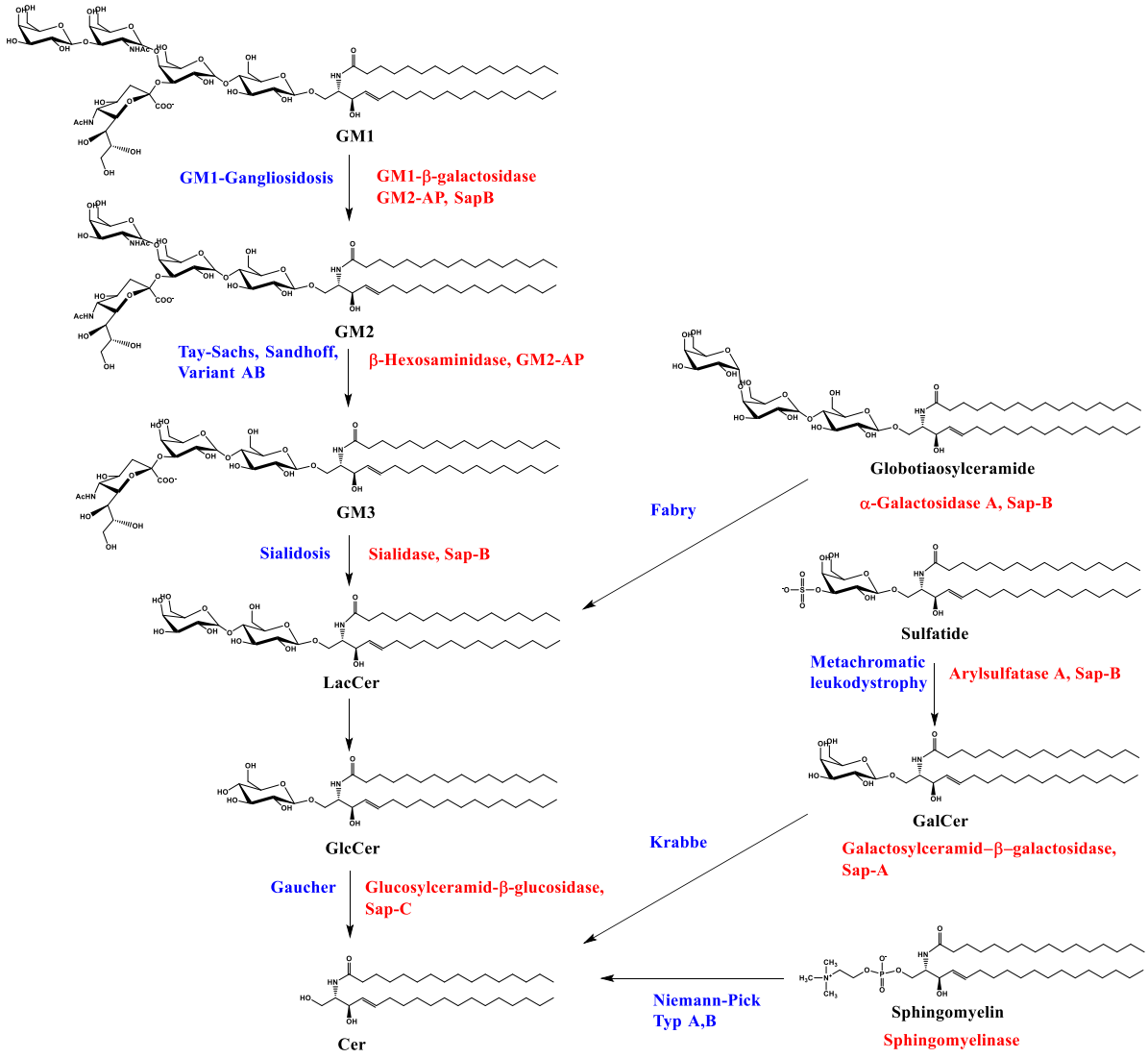

Figure 4: Pathway of lysosomal sphingolipid degradation (adapted from Sandhoff and Harzer (*7*))

The degradation of galactosylceramide (GalCer) is catalysed by galactosylceramide-β-galactosidase which requires Sap-A. The defective Sap-A results in an accumulation of GalCer and causes the late-onset form of Krabbe disease. The Sap-B is required for the degradation of sulfatide and globotriaosylceramide. The sulfatide degradation is catalysed by arylsulfatase A and is facilitated by Sap-B. Whereas the globotriaosylceramide is degraded by α-galactosidase A, but also with use of Sap-B. The defective Sap-B causes an accumulation of sulfatide, globotriaosylceramide and digalactosylceramide and leads to the atypical form of MLD

(metachromatic leukodystrophy). The degradation of glucosylceramide (GlcCer) is catalysed by glucosylceramide-β-glucosidase and mediated by Sap-C. The inherited deficiency leads to an accumulation of glucosylceramide and causes an abnormal juvenile form of Gaucher disease. The ceramide degradation is stimulated by Sap-D and catalysed by acid ceramidase. The deficiency of Sap-D causes an accumulation of ceramides with hydroxylated fatty acid chains. These ceramides are especially found in kidney and brain. The degradation of GM1 to GM2 by GM1-β-galactosidase requires GM2-AP or Sap-B. Furthermore anionic BMP stimulates the GM1 degradation. The deficiency of GM1-β-galactosidase leads to GM1-gangliosidosis and to an accumulation of galactose containing keratin sulfate and also oligosaccharides which leads to mucopolysaccharidosis IV B. The degradation of GM2 is achieved by the heterodimer β-hexosaminidase A (αβ) and GM2-AP. Tay-Sachs disease refers to inherited deficiencies in the HEXA gene causing the loss of β-hexosaminidase A (αβ) and S (αα) activity. The B1 variant of GM2-gangliosidosis is caused by mutations in HEXA gene, affecting the enzymes, but not the heterodimer formation of β-hexosaminidase A (αβ). Whereas Sandhoff disease is caused by mutations in HEXB gene, affecting the enzyme activity. The AB-variant of GM2-gangliosidosis is a very rare disease caused by the deficiency of GM2-AP. The deficiency of sphingomyelinase causes an accumulation of sphingomyelin and results as Niemann-Pick type A and B. Whereas Niemann-Pick type C is caused by the deficiency of NPC1 and NPC2, which are required for the transport of cholesterol out of the lysosomes (*3*, *7*, *8*). Each lysosomal storage disease caused by a specific enzyme deficiency is described in *2.5. Lysosomal storage diseases*, in detail.

2.4. Structure, function and formation of *lyso*-sphingolipids

Especially the "*lyso"* forms of sphingolipids have attracted significant interest over the last few years. In the *lyso*-sphingolipids the N-acyl- substituent is cleaved of and the results are the sphingoid bases with attached headgroups at position 1. Furthermore it has been proposed that *lyso*-sphingolipids are potential biomarkers for the diagnosis of lysosomal storage diseases and also for monitoring of treatment effects (*9*). About the biological function and origin of *lyso*-glycosphingolipids little is known, but they are found in tissues and blood and they are potential bioactive compounds (*6*). It is supposed to, that the pathogenesis of lysosomal storage disease, especially neural degeneration, depends on the accumulation of the cytotoxic *lyso*-sphingolipids (*10*). In figure 5 the accumulating *lyso*-sphingolipids of interest and their corresponding disorders, in blue, are shown.

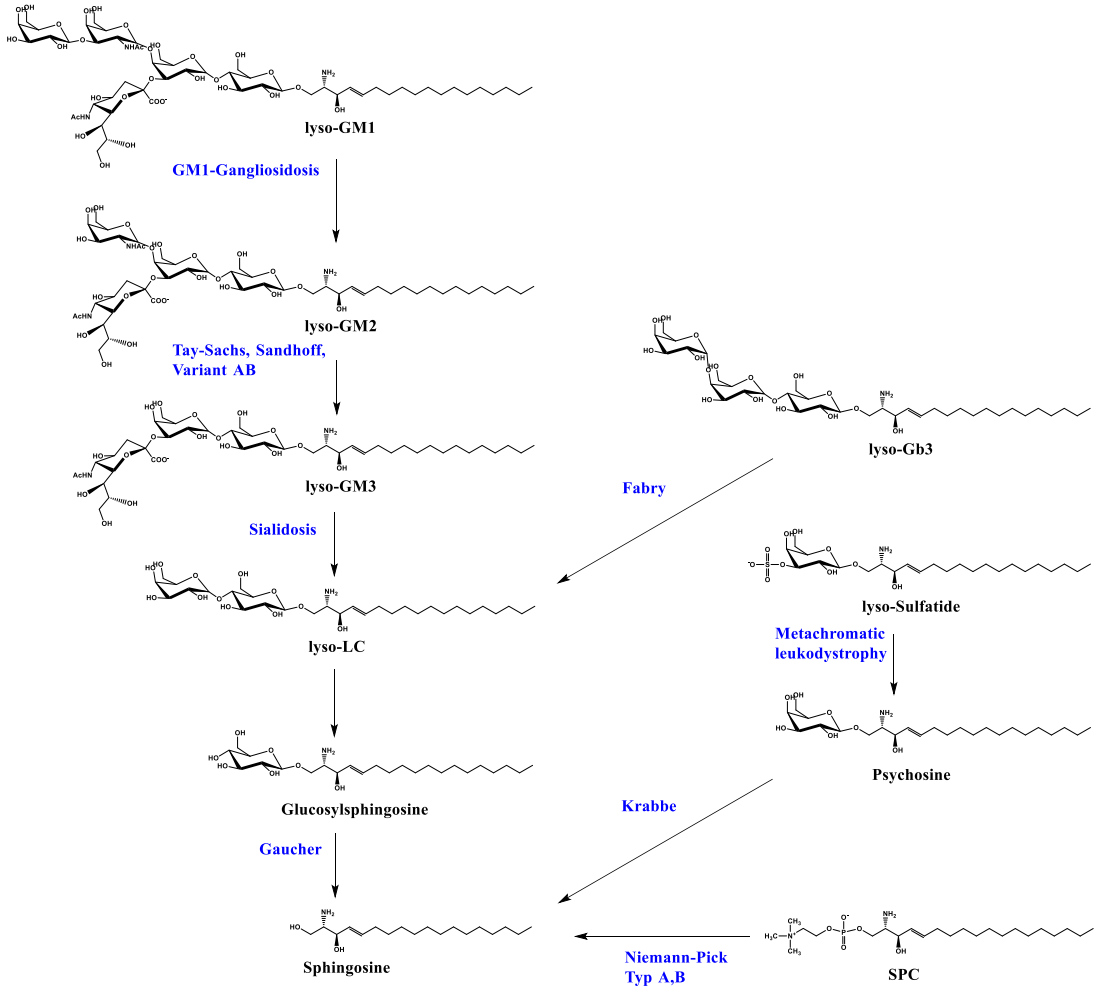

Figure 5: Lyso-sphingolipids and related lysosomal storage diseases (adapted scheme from Sandhoff and Harzer (7))

It has been shown, that the corresponding lyso-sphingolipids are accumulated in brain samples of patients with Tay-Sachs disease, GM1-gangliosidosis, metachromatic leukodystrophy and Niemann-Pick type C disease (*11*).

The *lyso*-GM1 level is elevated in patients with GM1-gangliosidosis. Whereas the levels of *lyso*-GM2 are elevated in plasma and brain of patients suffering from Tay-Sachs and Sandhoff disease (*10, 12*). However the origin of *lyso*-GM2 is not clear, it is associated with the enzyme activity of β-hexosaminidase A and also the pathogenesis of Tay-Sachs and Sandhoff disease is related to *lyso*-GM2. The *lyso*-GM2 level in human plasma increases, due to the activity loss of β-hexosaminidase. Therefore *lyso*-GM2 is supposed to be a potential biomarker for diagnosis of Tay-Sachs and Sandhoff disease (*10*).

The level of toxic *lyso*-Gb3 is elevated in plasma of patients suffering from Fabry disease. The *lyso*-Gb3 is the deacylated derivative of globotriaosylceramide (Gb3) and the pathogenesis of Fabry disease is associated with the accumulation of *lyso*-Gb3 in plasma (*13*). It has been proved that plasma *lyso*-Gb3 is a potential biomarker for monitoring of Fabry disease (*14*).

The *lyso*-sulfatide has been reported as a cytotoxic sulfatide derivative, which accumulates in tissues of metachromatic leukodystrophy (MLD) patients. The pathogenesis of MLD is also related to the accumulation of *lyso*-sulfatide (*15*). The demyelination of peripheral (PNS) and central nervous system (CNS) is associated with the accumulation of *lyso*-sulfatide in Schwann cells and oligodendrocytes. Furthermore it has been reported that *lyso*-sulfatide plays a role in the cellular calcium homeostasis. *In vitro* cell experiments showed, that elevated *lyso*-sulfatide levels cause cellular injuries and activation of intracellular proteases (*16*).

Psychosine is the neurotoxic derivative of GalCer and accumulates in different tissues, especially the brain, of patients suffering from Krabbe disease and causes demyelination and neurodegeneration. As mentioned it is the derivative of GalCer and it is generated either by galactosylation of sphingosine catalysed by UDP-galactose ceramide galactosyl transferase or by deacylation of GalCer catalysed by N-deacylase (*17*).

Glucosylsphingosine is a potential neurotoxic derivative of GlcCer and is elevated in the brain of patients with Gaucher disease (type 2 and 3). The accumulation of glucosylsphingosine is

associated with the neuronal dysfunction and destruction in Gaucher disease patients (*18*). As mentioned glucosylsphingosine is a derivative of GlcCer, but the biosynthesis of it is not quite clear. It is supposed to, that the biosynthesis of glucosylsphingosine is catalysed by glucosylceramide synthase. It has been proved that the of glucosylsphingosine is a potential biomarker for Gaucher disease (type 1) (*19*).

In Niemann-Pick type A, B and C the *lyso*-sphingomyelin level is elevated (*9*). The pathogenesis of Niemann-Pick disease may refers to the accumulation of the potent mitogen *lyso*-sphingomyelin in Niemann-Pick type A (*15*). In Niemann-Pick type A patients the accumulation of *lyso*-sphingomyelin in brain is associated with little or loss of sphingomyelinase activity. In Niemann-Pick type B patients elevated *lyso*-sphingomyelin levels are found in liver and spleen (*20*). It has been proposed that *lyso*-sphingomyelin is a potential biomarker for the diagnosis of Niemann-Pick type C patients (*21*).

2.5. Lysosomal storage diseases

The different lysosomal storage diseases (LSD) cover around 40 different diseases which are the result of a deficient enzyme, activator protein or transporter, required for the lysosomal catabolism. The lysosomal storage diseases belong to the rare disorders, because about one out of 7000-8000 newborns is affected (*15*). The lysosomal storage diseases are classified according to the accumulating substrate and to the pathway which is affected. According to the accumulating substrate the lysosomal storage diseases are classified in mucopolysaccharidosis, mucolipidosis, sphingolipidoses and glycoprotein and glycogen storage disease. Furthermore the lysosomal storage diseases vary in the progression and onset of the clinical symptoms. Therefore lysosomal storage disease are further distinguished in severe infantile, intermediate juvenile and mild adult form (*8, 22*).

In the focus of our interest are the following sphingolipidoses, GM1-gangliosidosis, GM2-gangliosidoses (Tay-Sachs disease, Sandhoff disease, AB-variant of GM2-gangliosidosis), Fabry disease, Gaucher disease, Metachromatic leukodystrophy, Krabbe disease and Niemann-Pick disease. They are inherited diseases caused by deficient genes encoding enzymes or proteins in the lysosomal sphingolipid catabolism. The sphingolipidoses are all autosomal recessive inheritances, except Fabry. The sphingolipidoses are characterised very well, because the degradation of glycosphingolipids occurs in a sequential manner, as described in *2.3. Glycosphingolipid degradation*. The sequential manner of glycosphingolipid degradation causes accumulation of substrates in the lysosomal pathway by a single affected enzyme or protein. The accumulation of a single substrate further leads to secondary storage products by co-precipitation of lipids, proteins and hydrophobic substances, this occurs for example in Niemann-Pick disease (*8, 15*). The single types of sphingolipidoses are described in detail below.

2.5.1. GM1-gangliosidosis

The sphingolipidose, GM1-gangliosidosis, belongs to the rare lysosomal storage disorder, because the incidence of affected newborns is 1 to 100 000-200 000 worldwide. The GM1-gangliosidose is an autosomal recessive disorder caused by the inherited deficiency of the lysosomal enzyme GM1-β-galactosidase (EC 3.2.1.23). On the chromosomal region 3p21.33, the GLB1 gene encodes for GM1-β-galactosidase. Until yet around 130 genetic lesions of the

GLB1 gene, including missense/nonsense mutations, duplications/insertions and insertions causing splicing defects, have been described. Together with cathepsin A, sialidase, N-acetylaminogalacto-6-sulfatase and so-called protective protein, GM1-β-galactosidase occurs as a lysosomal multienzyme complex. The degradation of the ganglioside GM1 to GM2 is catalysed by GM1-β-galactosidase and requires GM2-AP or Sap-B. The inherited deficiency of the enzyme GM1-β-galactosidase primarily leads to an accumulation of the ganglioside GM1, but also to an accumulation of oligosaccharides from glycoproteins, glycolipid GA1 and keratin sulfate. For example the accumulation and storage of GM1 in neurons causes the degradation of the nervous system (*15, 23*). According to the accumulations in different tissue, especially the central nervous system (CNS), the following clinical manifestations result (*24*).

There are three different clinical forms of GM1-gangliosidosis according to their onset and progression described:

- Type 1 (infantile): The infantile form is characterised by psychomotor regression and has its onset at the age of 6 months. Further symptoms are cherry red spot, visceromegaly, skeletal and facial abnormalities.
- Type 2 (late infantile/juvenile): The late infantile and juvenile form has its onset at the age between 7 months and 3 years and starts with slowly progressive neurological signs. These include muscle weakness, early locomotor problems, lethargy, seizures, strabismus and terminal bronchopneumonia.
- Type 3 (adult): The adult form has its onset at the age between 3-30 years and is the mildest phenotype. The characteristics of the adult form are dystonia, cerebellar dysfunction, short stature, slurred speech and mild vertebral deformities (*23*).

2.5.2. GM2-gangliosidoses

The different GM2-gangliosidoses are caused by the deficient lysosomal enzyme β-hexosaminidase (EC 3.2.1.52) or activator protein (GM2-AP). The ganglioside, GM2, degradation is catalysed by β-hexosaminidase and requires GM2-AP. The β-hexosaminidase cleaves the β-glycosidic linkage between N-acetylgalactosaminyl and the galactose residue. The β-hexosaminidase occurs as a homo- or heterodimeric protein consisting of two subunits, α and β. The HEXA gene and HEXB gene encode for the subunits α and β. Due to the combination of the two subunits three different β-hexosaminidase dimers are formed. The terminal N-

acetylgalactosamine and N-acetylglucosamine residue, β-glycosidically linked to un- and negatively- charged glycoconjugates is cleaved of by the heterodimer β-hexosaminidase A (αβ). Whereas the homodimer β-hexosaminidase S (αα) catalyses the degradation of the ganglioside GM2, sulfated glycolipids and glycosaminoglycans. The degradations of uncharged glycolipids and oligosaccharides with terminal residues of N-acetylhexosamines are catalysed by the homodimer β-hexosaminidase B (ββ). Due to the deficiency, either in one of the α- or β-subunit or the GM2-AP, three different variants of GM2-gangliosidoses occur.

Tay-Sachs disease (B-variant): In Tay-Sachs disease the α-subunit of the lysosomal enzyme β-hexosaminidase is affected. The deficiency of the α-subunit leads to defective β-hexosaminidases A and S, but the β-hexosaminidase B is fully functional.

There are four different clinical forms of GM2-ganglisidosis B-varianat, according to their onset described:

- Infantile form: The affected newborns are not showing visual symptoms at birth. The infantile form, known as Tay-Sachs disease has its onset at the age between 3-7 months and motor weakness is one of the first symptoms. Later on visual symptoms appear and others like hypotonia, weakness, decreasing attentiveness and poor head control are observed. In Tay-Sachs disease also the cherry red spot is observed. At the age of about 10 months visual, mental and motor abilities decrease rapidly. The affected children die at the age between 2-4 years.
- Juvenile form: The juvenile form has its onset at the age between 2-6 years and the first noted symptoms are motor symptoms. In the disease progression the following symptoms can occur, increasing spasticity, loss of speech and vision, seizures and progressive dementia. The affected children die at the age between 10-15 years.
- Chronic form: The chronic form has its onset at the age between 2-5 years and occurs with abnormalities in posture and gait. In the disease progression neurological symptoms occur, but verbal and mental intelligence is not affected. The life expectancy is about 40 years.
- Adult form: In the adult form patients suffer from neurological disorders.

Sandhoff disease (0-variant): In Sandhoff disease the β-subunit of the lysosomal enzyme β-hexosaminidase is affected. The deficiency of the β-subunit leads to defective β-hexosaminidases A and B, but the β-hexosaminidase S is fully functional.

There are three different clinical forms of Sandhoff disease, according to their onset described:

- Infantile form: The infantile Sandhoff disease shows similar pathological and clinical characteristic as Tay-Sachs disease. Furthermore mild forms of bone deformities and organomegaly can occur.
- Juvenile form: The juvenile form has its onset at the age between 3-10 years and shows symptoms like cerebellar ataxie, slurred speech and psychomotor retardation.
- Adult form: The adult form shows similar symptoms as Tay-Sachs, however with delayed onset.

GM2-gangliosidosis (AB-variant): A deficiency in the GM2-activator protein leads to the AB-variant of GM2-gangliosidosis. The β-hexosaminidases A, B and S are intact, but nevertheless an accumulation of the ganglioside GM2 occurs. The clinical symptoms of AB-variant are similar to Tay-Sachs, however with delayed onset (*7, 10, 15*).

2.5.3. Fabry disease

The sphingolipidose, Fabry disease, belongs to the rare lysosomal storage disorder, because the incidence of affected newborns is 1 to 40 000-117 000 worldwide. Fabry disease is a X-chromosomal linked disorder caused by the inherited deficiency of the lysosomal enzyme α-galactosidase A. The GAL gene encodes for α-galactosidase A and until yet around 400 genetic lesions of the GAL gene, including missense or nonsense mutations and point mutations, such as deletions and insertions, have been described. The globotriaosylceramide (Gb3) degradation is catalysed by α-galactosidase A and requires Sap-B. A reduced or absent α-galactosidase A activity leads to accumulation of Gb3 and related glycosphingolipids in the lysosomes. During early childhood the following symptoms occur temperature intolerance, angiokeratomas, gastroindestinal problems and burning sensations in hands and feet. Symptoms occurring in late adolescence and early adulthood are cardiac hyperdrophy and arrhythmia, proteinuria and glomerulosclerosis and other cardiovascular diseases. Due to Fabry is a X-linked disease, heterozygous females have a higher residual activity of α-galactosidase A and the symptoms are

attenuated in comparison to men. The Fabry disease has its mean onset at the age between 3-10 years for males and 6-15 years for females (*15, 25*).

2.5.4. Gaucher disease

The lysosomal storage disorder, Gaucher disease is the most common sphingolipidose. The Gaucher disease is caused by the inherited deficiency of the lysosomal enzyme glucosylceramide-β-glucosidase (EC 3.2.1.45). On the chromosomal region 1q21-22, the GBA gene encodes for glucosylceramide-β-glucosidase. Until yet around 300 genetic lesions of the GBA gene, including point mutations as deletions and insertions, frame-shift mutations, recombinant alleles and site mutations, have been described. The glucosylceramide degradation is catalysed by glucosylceramide-β-glucosidase and is activated by Sap-C and BMP. The deficiency of glucosylceramide-β-glucosidase causes an accumulation of glucosylceramide in plasma, spleen, liver and bone marrow. The complete loss of glucosylceramide-β-glucosidase activity leads to severe impairment of skin function and the results are "collodion babies".

There are three different types of Gaucher disease which are distinguished according to the presence or absence of neurological involvement.

- Type 1 (non-neuronopathic): The type 1, is the attenuated and most common form of Gaucher disease. The incidence of affected newborns is 1 to 50 000-200 000 worldwide and the life expectancies of affected patients are between 6-80 years.
- Type 2 (acute neuronopathic): The type 2, is the acute form of Gaucher disease and is a rare panethnic disease with an early onset and involvement of the nervous system. The life expectancies of affected patients are less than two years.
- Type 3 (sub-acute neuronopathic): The type 3, the sub-acute or juvenile form, is the intermediate form of Gaucher disease type 1 and 2, mainly found in Northern Swedish population. The onset of type 3 is later and the neurological symptoms have a slower development, furthermore the life expectancies are a few years to 4th decade of life.

In all three types the patients show symptoms like anemia, thrombocytopenia, hepatosplenomegaly and bone damage, in various severity (*15, 26*).

2.5.5. Metachromatic leukodystrophy (MLD)

The sphingolipidose, metachromatic leukodystrophy, belongs to the rare lysosomal storage disorder, because the incidence of affected newborns is 1 to 40 000-100 000 worldwide. The metachromatic leukodystrophy is an autosomal recessive disorder caused by the inherited deficiency of the lysosomal enzyme arylsulfatase A. On the chromosomal region 22q13.33, the ARSA gene encodes for arylsulfatase A. Until yet around 60 genetic lesions of the ARSA gene have been described. The degradation of sulfatide to galactosylceramide and sulfate is catalysed by arylsulfatase A and requires Sap-B. The inherited deficiency of the enzyme arylsulfatase A leads to an accumulation of sulfatide in several tissues like white matter of brain, myelin sheaths, kidney tissue and peripheral nervous system. The clinical manifestation of MLD are caused by the accumulation in the myelin sheaths which leads to demyelination. The inherited deficiency of Sap-B is quite rare, but shows similar symptoms like MLD with enzyme deficiency.

There are three different clinical forms of MLD, according to their onset and are all characteristic by a variety of neurological symptoms, described:

- Late infantile form: The late infantile form of MLD has its onset at the age between 6 months and 4 years with a rapid progression of psychomotor regression. The patients die within five years after occurrence of the first symptoms. First symptoms of metachromatic leukodystrophy are mental regression, hypotonia and unsteady gait. Further symptoms are blindness, loss of speech, peripheral neuropathy quadriparesis and seizures. In the final stage of the disease before death, the child is bedridden and loses contact with its surrounding.
- Juvenile form: The juvenile form of MLD has its onset at the age between 4-16 years with a slower progression than the infantile form, at the beginning of the disease.
- Adult form: The adult form of MLD has its onset after puberty up to the 6th decade of life. This form of MLD is less frequent in comparison to the two others.

In both, juvenile and adult form, the school and job performances show a gradual deterioration. The performance deteriorations occur with behavioural and emotional disturbances or psychiatric symptoms especially in the adult form. Further symptoms are incontinence, gait clumsiness and optic atrophy. The patients reach vegetative state in the final stages of the disease (*15, 27*).

2.5.6. Krabbe disease

The sphingolipidose, Krabbe disease, belongs to the rare lysosomal storage disorder and is an autosomal recessive disorder caused by the inherited deficiency of the lysosomal enzyme galactosylceramide-β-galactosidase (EC 3.2.1.46). On the chromosomal region 14q31, the GALC gene encodes for galactosylceramide-β-galactosidase. Until yet around 128 genetic lesions of the GALC gene including deletions, frame shift and missense mutations, have been described. The degradation of galactosylceramide to galactose and ceramide is catalysed by galactosylceramide-β-galactosidase and is stimulated by Sap A and Sap B. The inherited deficiency of the galactosylceramide-β-galactosidase leads to an accumulation of enzyme substrate, galactosylceramide, but also to the storage of neurotoxic galactosylsphingosine (psychosine) in macrophages (globoid cells) and in neural cells especially in Schwann cells and oligodeudrocytes. The characteristic pathological features of Krabbe disease are caused by impaired galactosylceramide degradation and accumulation of psychosine. The clinical manifestations of Krabbe disease occur due to extensive demyelination, multinucleated macrophages in brain and white matter and gliosis.

There are two different clinical forms of Krabbe disease, according to their onset described:

- Infantile form: The infantile form of Krabbe disease has its onset at the age between 3 to 6 months with hypersensitivity or irritability of external stimuli and severe motor and mental deteriorations and growth retardation occur. Furthermore patients become flaccid, blind, deaf and hypotonic. Patients suffering from infantile Krabbe disease die within the first two years of life.
- Late onset form: The late onset form of Krabbe disease has its onset at the age between a few years, at the age children start walking, but up to 73 years. The clinical symptoms are more heterogeneous in comparison to the infantile form and are less severe. Common symptoms are psychomotor retardation, spastic paraparesis, blindness and dementia (*15, 17*).

2.5.7. Niemann-Pick disease (NPD)

The sphingolipidose, Niemann-Pick disease type A and B, belongs to the rare lysosomal storage disorder and is an autosomal recessive disorder caused by the inherited deficiency of the lysosomal enzyme acid sphingomyelinase (EC 3.2.4.12). On the chromosomal region 11p15.4

the SMPD1 gene encodes for acid sphingomyelinase. Until yet around 120 genetic lesions of the SMPD1 gene, including point mutations (missense and non-sense), small deletions and splicing abnormalities, have been described. The degradation of sphingomyelin to phosphorylcholine and ceramide is catalysed by acid sphingomyelinase. Sphingomyelin is an important membrane component and also a component of the myelin sheath. The inherited deficiency of the acid sphingomyelinase leads to an accumulation of sphingomyelin and other lipids in characteristic "Niemann-Pick-cells", with mulberry appearance. These cells are present in liver, lymph nodes, spleen, lungs, bone marrow and adrenal cortex. Furthermore the deficiency of acid sphingomyelinase, leads to a elevation of BMP, *lyso*-sphingomyelin, cholesterol, glucocerebroside, lactosylceramide and ganglioside. The elevation of these substrates is more common in NPC type C. The Niemann-Pick disease type C is caused by the inherited deficiency of NPC1 and NPC2. The cholesterol is shuttled out of the lysosomes mediated by NPC1 and NPC2. The deficiency of NPC1 and/or NPC2 leads to an accumulation of intracellular cholesterol and secondary to an accumulation of sphingomyelin. The clinical symptoms in type A and B occur according to the lipid abnormalities in the plasma membrane.

As mentioned three different types of Niemann-Pick can occur:

- Niemann-Pick A: The NPD type A is a fatal disorder with a life expectancy of 2-3 years. The affected newborns show a normal appearance at birth, but the first symptoms appear in the first few months after birth. These symptoms include muscular weakness, hepatosplenomegaly, hypotonia, moderate lymphadenopathy and cherry-red spot. Until the age of six months the developmental process is normal. From about 6 to 15 months the developmental progress like growth and body weight decreases and also psychomotor retardation occurs. In the final state patients loose contact with their environment.
- Niemann-Pick B: The NPD type B varies in comparison to type A in onset and progression and the patients live into adulthood. The type B shows little or no involvement in the nervous system, but liver or/and spleen are enlarged. Symptoms are hepatosplenomegaly, elevated serum triglyceride and LDL-content and patients suffer from progressive pulmonary function problems.

- Niemann-Pick C: The clinical symptoms of NPD type C are extremely heterogeneous. The type C form of NPD has its onset at a few days after birth to a age over 60 years. The onset of the disease most often is between 10-25 years. Clinical manifestations contain visceral involvement (liver, lung and spleen) and neurological or psychiatric manifestations occur at different times. Patients suffer from neurological disorders like cerebellar ataxia, dysphagia, dysarthria, vertical supranuclear gaze plasy and progressive dementia. A common characteristic in late onset patients are psychiatric disturbances (*15, 28, 29*).

2.6. Pathogenesis and therapeutic approaches

Pathogenesis of sphingolipidoses

As described before the sphingolipidoses show high phenotypic variability in onset, development and symptoms, even within one disease. The pathogenesis of the disease is determined by the intra lysosomal storage of glycosphingolipids in tissue and organs. This storage causes secondary response which can lead to tissue damage or cell death. Another factor for pathogenesis is the residual enzyme activity of deficient lysosomal enzymes. The residual activity determines the onset and severity of the lysosomal storage disorder. Whereas a complete deficiency of degrading enzymes leads to an early and sever form of the disease, however, a little residual activity leads to a later onset and attenuated form of the disease. Furthermore the pathogenesis is influenced by the nature of accumulating enzyme substrates. For example, formation of bioactive or toxic substrates like psychosine, glucosylsphingosine, *lyso*-sulfatide, SPC and *lyso*-glycosphingolipids, influence the pathogenesis of the disease. Another factor is inflammation which plays an important role in neuronal death, probably because of distribution of the blood brain barrier. The sphingolipidoses are diagnosed according to characteristic pathological manifestations and clinical symptoms, measurement of enzyme activity and storage compound analysis (*15, 30*).

Therapeutic approaches

For the treatment of lysosomal storage diseases the pathogenesis of each sphingolipidose is very important. Due to the substrate storage within the lysosomal compartments many secondary cellular responses occur. The storage leads to failures in housekeeping cellular pathways and functions, including modulation of receptor response and signal transduction cascades, reporter activation by non-physiologic ligands, impaired intracellular trafficking of vesicles, activation of inflammatory response, membrane-bound proteins and membranes. All cellular processes involved in the pathogenic cascade of lysosomal storage diseases are therefore possible targets for therapy. Today's therapeutic approaches to treat lysosomal storage diseases are based on the elevation of the missing enzyme in cell and tissue (*30*). The following approaches are able treatments for lysosomal storage diseases:

1. Hematopoietic stem cell transplantation (HSCT):

In the treatment of lysosomal storage diseases Hematopoietic stem cell transplantation (HSCT) was the first therapeutic approach. In HSCT, as a treatment agent, hematopoietic stem cells from a healthy donor are used. A successful treatment with HSCT is achieved through two steps. The first one is to reoccupy specific tissue with healthy donor cells. The second one is, that the healthy donor cells have to secrete functional enzymes into the extracellular space and blood. Then the functional enzymes are taken up by recipient cells and there they cross-correct the defective enzyme. The HSCT is limited to a few lysosomal storage diseases, like mucopolysaccharidosis (MPS type I), Krabbe disease (late-onset) and metachromatic leukodystrophy (attenuated form). The advantage of HSCT is that the donor cells are able to reach the brain and improve the neurocognitive function. The disadvantages are, that the availability of suitable donors is limited and just a few disorders are treatable with HSCT (*30*).

2. Enzyme replacement therapy (ERT):

In the treatment of lysosomal storage diseases with enzyme replacement therapy, human recombinant lysosomal enzymes are periodically intravenous infused. The injected recombinant enzymes are spread to the different tissues, where they are internalised by the cell. After internalisation the recombinant enzymes are targeted to the lysosomal compartment, where the defective enzyme is replaced. The ERT shows success in the treatment of lysosomal storage diseases like Gaucher disease, Fabry disease and Pompe disease (type I, II, VI). The limitations of ERT are the high costs of therapy and the bioavailability. Another limitation of ERT is the impaired membrane diffusion of recombinant enzymes, because the large recombinant enzymes cannot freely diffuse across cell membranes. This may causes a low concentration of recombinant enzymes in the target tissues and therefore no therapeutic effect occurs. Furthermore these large recombinant enzymes are unable to pass the blood-brain barrier. Therefore they do not reach the CNS, which is of great clinical relevance, because in pathogenesis of most lysosomal storage diseases the CNS and neurodegeneration plays an important role (*30*).

3. Pharmacological chaperone therapy (PCT):

The pharmacological chaperone therapy (PCT) in the treatment of lysosomal storage diseases recently gained attention. In PCT small-molecule ligands, the pharmacological chaperones,

interact with misfolded proteins. This chaperon-protein interaction causes a conformation correction and a stability enhancement of the misfolded protein. The first proposed treatment with PCT was for Fabry disease, later on for Gauche and Pompe disease too. In comparison to ERT, PCT has following advantages: may administered orally, is non-immunogenic, non-invasive treatment and do not need to be shuttled to cells through a pathway. Furthermore it is expected that the small molecule chaperones may freely diffuse through membranes and reach tissues which are hardly accessible for recombinant enzymes, for example the CNS. On the other hand most pharmacological chaperones are potential competitive inhibitors, because they interact with the active site at the target enzymes (*30*).

4. Gene therapy (GT):

With gene therapy (GT) a complete and permanent correction of the defective enzymes in lysosomal storage diseases is possible. In GT the activity of the defective enzyme in the cell is increased or restored by supplying functional enzymes. This is achieved through shuttling of normal copies of the defective gene to recipient cells. There, according to the normal gene copies, functional enzymes are synthesised. The gene transfer of the normal gene copies is achieved with different viral vectors. The potential for long-term expression of the therapeutic enzyme is an advantage of GT. The disadvantage of GT is the gene transfer with viral vectors, because it is too less known about the possibility of carcinogenesis and the viral vector safety in human (*30*).

5. Substrate reduction therapy (SRT):

The SRT is based on reduction of substrate storage in the lysosomes by inhibition of specific steps in the substrate biosynthesis. The inhibition of specific steps in the pathway are achieved by small-molecule inhibitors which are inhibiting the enzymes required for substrate biosynthesis. The treatment of Gauche disease (type 1) and Niemann-Pick (type C) with SRT has been approved for clinical use. Gaucher disease, for example, can be effectively treated with N-buthyldeoxynojirimycin (Miglustat). Furthermore, in preclinical studies, it has been proposed that SRT also can be used for the treatment of other lysosomal storage disorders as Sandhoff and Fabry (*30*).

2.7. LC-MS (Liquid chromatography-mass spectrometry)

LC-MS is nowadays a routine method which combines two very important techniques, liquid chromatography and mass spectrometry. The technique can be applied to a wide range of molecules and in one single run a lot of different compounds can be measured. It was always desirable to couple the liquid chromatography to MS, because MS is very sensitive and highly specific (*31*). A lot of effort was needed to combine these two different techniques, because the two techniques are working in two different phases. On one hand there is the LC which is based on liquid phase separation and on the other hand there is the MS which is analysing in gas phase. The problem to handle was to convert the eluate with a high flow rate and pressure from LC into an appropriate gas flow for MS. But also the following problems, reduce the eluate volume, convert the analyte into gas phase, reduce the pressure and ionization of the analyte had to be taken under control. The solution for these problems was the invention of the ESI (electrospray ionization) (*32*).

2.7.1. LC (liquid chromatography)

In most cases the LC which is used for separation nowadays, is a high performance liquid chromatography (HPLC). The basically design of a HPLC consists of a mobile phase in an eluent reservoir, a degasser, a pump, an injector, a pre-column, a column and a detector.

The mobile phase elutes the substances from the column and is selected according to the separation problem. It is important that the mobile phase has high purity without any particles. The containing air bubbles in a mobile phase are removed by a degasser. It is necessary to remove the air, because even little bubbles at the pump piston drastically influence the pulsation of the pump. The pump is needed to deliver the mobile phase in a constant flow through the HPLC system. Therefor constant pumps without pulsation are needed to get an accurate baseline. There are two different ways for elution, the isocratic and the gradient elution. In the isocratic elution the chemical composition of the mobile phase remains the same over the whole run of separation. This is a useful technique for an easy separation problem with quite similar, but well separating components. For a difficult separation problem with lots of different components with various polarities it is better to use a gradient elution. If an isocratic method is used for such a problem, the compounds which are retarded to the stationary phase are eluting very slowly and result in a broad peak. Then the gradient elution is the method of choice. In the

gradient elution two or three different mobile phases are used and the composition changes during the elution. There are different ways to perform a gradient elution. It can be performed as a convex, concave, linear or a step wise gradient. The injector is needed to bring a define volume of the sample into the system without pressure reduction. Therefor a microliter injection syringe and a six-way valve is used. Sometimes a pre-column is used to protect the column from contaminations. The analytes are separated due to interactions with the stationary phase in the column and the mobile phase. The specification of packed columns are a small particle size and a high packaging density. The analytes are in interaction with the mobile phase and the stationary phase. The main HPLC principle of separation is adsorption. The stationary phase is either a polar normal- phase or a nonpolar reversed-phase. The reverse-phase column is packed with modified silica gel (*33*).

As mentioned above the separation occurs due to the interactions of the analytes with the mobile phase and the stationary phase. To get a chromatogram with well-shaped peaks the relation between the packaging of the column (stationary phase) and the flow rate of the mobile phase is important. This relation is described in the Van-deemter- equation (1) or can be seen in the Van-deemter-curve (figure 6).

$$H_{(HETP)} = A + \frac{B}{u} + C * u \qquad (1)$$

$H_{(HETP)}$… height equivalent to a theoretical plate
A…Eddy-diffusion
B…longitudinal diffusion
C…mass transfer
u…flow rate

The Van-deemter-curve, shown in figure 6, is a result of the following factors. The Eddi-diffusion (A) is the different way of diffusion, of two various components, through the column. This diffusion is independent from the flow rate, but is influenced by the particle size and the packaging density. A further factor is the longitudinal diffusion (B). The longitudinal diffusion is the diffusion of the analytes in the mobile phase in flow direction. This diffusion drops with higher flow rate. That factor is more important for GC (gas chromatography) then for LC. The

third factor is the mass transfer (C). The mass transfer is the diffusion of analytes between the column material and the mobile phase. This factor is linear by an increasing flow rate. The Van-deemter-curve (D) is the result of these three factors (*33*).

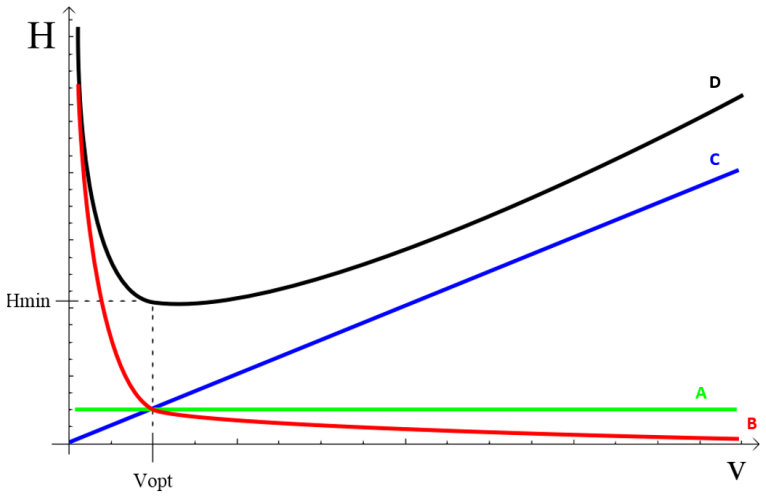

Figure 6: Van-deemter-curve

A chromatogram with well-shaped peaks results with an accurate flow rate adapted to the packaging material of the column. If the flow rate is too high, the analytes cannot interact with the stationary phase. The result is a poor separation of the analytes. Also a poor separation results if the flow rate is too low as it can be seen in figure 6. If the flow rate is very low the diffusion is getting higher which also results in a poor separation of the analytes. To receive a chromatogram with well-shaped peak of the analytes a detector is needed. There are many different types of detectors, but they can be divided into three main groups. The first group are the non-selective detectors like the conductivity, the refraction index, the light scattering and the UV-VIS detector. The second ones are the selective detectors like AAS (atom absorption spectroscopy), ICP-OES (inductively coupled plasma optical emission spectroscopy) and ICP-MS (inductively coupled plasma mass spectroscopy). At least there are the molecule selective detectors like ESI-MS (electrospray ionization mass spectrometry), which are discussed in *2.7.4. Design of a HR-MS-device* (*33*).

2.7.2. Column

The theoretical background of the used columns for method development are briefly described in the following chapter.

HILIC (Hydrophilic interaction liquid chromatography)

The hydrophilic interaction liquid chromatography (HILIC) is an alternative to the normal-phase liquid chromatography (NP-LC). As in NP-LC, it is possible to separate small polar molecules with HILIC, which have poor retention in reversed-phase liquid chromatography (RP-LC). Whereas the mobile phases used for separation with HILIC are similar to the mobile phases used in RP-LC. In comparison to RP-LC, the mobile phase are used vice versa in gradient elution with HILIC. Whereas a gradient elution in HILIC starts with a rather non-polar organic mobile phase and the polarity is increased by a higher water content, to elute the polar analytes.

The present separation mechanism proposed for HILIC is partitioning. The separation is achieved through the different distribution of the analytes in the acetonitrile rich mobile phase and the aqueous-rich layer absorbed onto the stationary phase. That means that the more hydrophilic components get immobilized on the stationary phase because of partitioning, whereas more hydrophobic analytes are shifted to mobile phase. To summarize the partitioning in the different phases depends on the polarity of the analytes. In HILIC not only the partitioning mechanism plays an important role also the interactions like hydrogen donor and electrostatic interactions. (*34*).

PGC (porous graphitic carbon)

The porous graphitic carbon (PGC) is as crystalline material consisting of graphitic sheets, where the carbon atoms are hexagonally arranged and the sheets interact through van der Waals interactions. The strength of analyte interactions with the graphitic surface is based on the polarisability of the graphitic surface and the analyte, but also the distance of the analyte to the surface. That means that the strength of interaction depends on the electronic distribution and the shape of the analyte. Both analytes, polar and non-polar, are retained on the surface of PGC, this is due to polarisability of the graphitic surface. As mentioned the shape of the analytes also plays a role in the strength of interaction. The planar shaped molecules are aligned closer to the planar graphitic surface than non-planar analytes, so the strength of interaction is higher. Due to the retention of polar and non-polar molecules the PGC columns are used for both RP-LC and NP-LC (*35*).

2.7.3. ESI (electrospray ionization)

The ESI (electrospray ionization), a well-known atmospheric pressure ionization method which is primary used for the coupling of LC and MS. The electrospray ionization is a soft ionization method and transfers the ions from the liquid phase into the gas phase. It is a suitable method to analyse big, non-volatile molecules which pick up charge easily, like proteins and nucleic acid.

The analytes separated with liquid chromatography are located in liquid phase. For MS analysis ions in gas phase are needed. To generate ions in gas phase the following design of an ESI-source is used nowadays.

The first part of an ESI is the ESI-nozzle, an electroconductive capillary with a potential of 3-4 kV, which produces an aerosol spray at atmospheric pressure, containing electrically charged droplets in micrometer size. Between the capillary and a counter electrode an electric field results. When the changed droplets from the capillary are entering the electric field the aerosol spray occurs. This occurrence is based on the Taylor-cone formation and the Coulomb-explosion. A Taylor-cone is formed because of charge separation in the capillary. According to the voltage applied we distinguish between positive-ion-mode and negative-ion-mode. In the positive-ion-mode the capillary is positively charged, the anions are oxidized at the capillary wall and the cations form positive charged droplets. In the negative-ion-mode it is vice versa, the capillary is negatively charged and the cations are reduced at the capillary wall and the anions form negative charged droplets. The charged droplets undergo a desolvation in a nitrogen flow towards direction and began to shrink, because of heat and nitrogen. The charged droplets shrink till the Rayleigh-limit. At the Rayleigh-limit the charges on the droplet surface get to close to each other and the Coulomb-explosion occurs.

The release of ions is described in two different models the CRE (charged residue model) and the IEM (ion evaporation model). The CRE is the older one of these two models. This model describes the gradually loss of solvent of the little droplets caused by Coulomb-explosion, which contain only one analyte molecule. In the end the droplets containing only one molecule undergo a complete desolvation. The remained charge of the droplet is supposed to be transferred to the single molecule in the droplet. Whereas the newer model, the IEM postulates an ion evaporation. In that process the ions are produced by evaporation of the larger charged droplets (*36*).

2.7.4. Design of a HR-MS-device

The first part in an HR-MS coupled to LC is the ESI-sprayer, which was discussed before. The ESI is nowadays in most systems located in an off axis design in front of the ion inlet. The off axis geometry has the advantage that contaminations are adsorbed at the ion inlet and don´t get through the transfer line. Especially non-volatile compounds like salts and organic compounds from blood or urine shouldn´t reach the S-lenses in the desolvation zone.

The ion spray is produced at atmospheric pressure, whereas in the mass analyser a high-vacuum system is needed. The high-vacuum is gradually realised with differential pumping stages. The first pumping stage is after the transfer line. So the transfer line is the separation between atmospheric pressure and vacuum (~ 10^2 Pa). At this first stage most of the ions are drained and don´t get through the S-lenses or skimmer, used in early days. In the S-lenses the ions are focused and transferred to the next stage. In this next stage or second stage high-vacuum (10^{-3}-10^{-4} Pa) is needed. In the second pumping stage a quadrupole is located most of the time. This quadrupole could function as mass analyser or as an ion selector for further mass analyser like TOF (time of flight) or orbitrap (*36*).

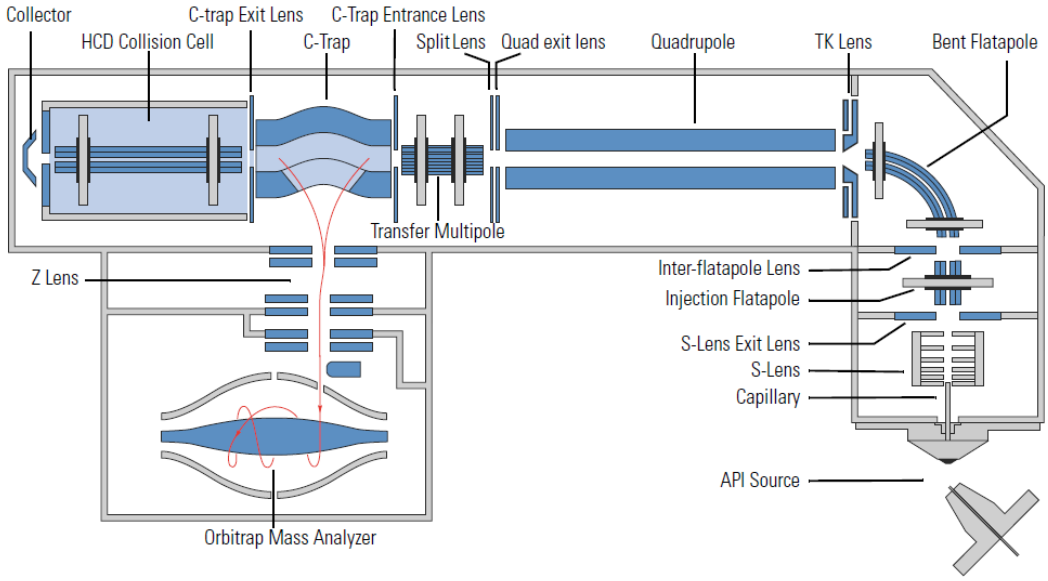

Figure 7: Scheme of a Thermo Fisher Scientific orbitrap (graphic from Thermo Fisher Scientific)

There are many different mass analysers available, but the following experiments were done with a high-resolution mass spectrometer, an orbitrap mass analyser from Thermo Fischer Scientific. Therefor we have a look at the orbitrap in detail. With the orbitrap, an ion trap, an

exact mass determination, with a high resolution is possible. In contrast to other ion traps, the orbitrap captures the moving ions with an electrostatic field and no frequency stimulation or magnet is needed.

In figure 7 the design of the orbitrap mass analyser can be seen. The orbitrap consists of a two-part, barrel-like outer electrode and in the inside there is a spindle-shaped central electrode. The entering ions oscillate in a helical way around the central electrode, because of the axial electrostatic field in the trap. The barrel-shaped outer electrode is isolated with a ceramic ring, which separates it into two parts. The rotation frequency of the harmonic axial oscillation of ions are detected with image stream detection, which is coupled to both outer electrodes. The image stream is recorded and converted to a signal with FFT (fast Fourier transformation). The result of the signal is an exact m/z-value, because the frequency of the axial movement is conform to every m/z-value. The design described so far is not yet a high resolution detector, because the frequency of ions in the orbitrap depends on the entering velocity of the ions. To measure an exact m/z-value the right ion injection and an ultra-high vacuum is needed. For the right ion injection the following have to be considered: the injection angle, diffusion angle and the position where the ions reach the electrode. Also the initially velocity of the ions and the velocity distribution is important. Furthermore the injection moment, the injection duration and the dosage of ions have to be considered. Therefore an ultra-high vacuum and a bent RF-quadrupole (radio-frequency quadrupole) is needed. Because of its bent it is called C-trap. In the C-trap nitrogen is use to catch the ions, cool them and slow them down. These slowed down ions are ejected in form of an ion bundle with high voltage pulses from the C-trap to the orbitrap. Because of the electrical potential of the central electrode the ion bundle get caught and start there circular movement around the central electrode. The different m/z-frequencies are measured and transformed into a signal (*37*).

3. Material and Methods

3.1. Material

The standards and internal standards (IST) used for method development and quantification are shown in table 1 below and were all from Matreya LLC.

Table 1: Standards and internal standards from Matreya LLC

Substance	Cat. No.	Lot.
Psychosine (PSY) (free amine form); semisynthetic, bovine	1305	23834
Glucosylsphingosine (GLY); semisynthetic, bovine buttermilk	1306	24201
Glucosylsphingosine (GLY plant); semisynthetic, plant	1310	23798
***lyso*-Globotriaosylsphingosine (*lyso*-Gb3)**; semisynthetic, porcine RBC (red blood cells)	1520	-
***lyso*-Lactosylceramide (*lyso*-LC)**; semisynthetic, bovine buttermilk	1517	-
***lyso*-Sphingomyelin (SPC)**; semisynthetic, bovine buttermilk	1321	24175
***lyso*-Sulfatide** (NH_4^+ salt); semisynthetic, bovine	1904	20822
***lyso*-Monosialoganglioside GM1 (*lyso*-GM1)** (NH_4^+ salt); semisynthetic, bovine	1518	22482
N-omega-CD_3-Octadecanoyl monosialoganglioside GM1 (D_3GM1) (NH_4^+ salt); semisynthetic, bovine	2050	23054
Monosialoganglioside GM2 (NH_4^+ salt); natural, human Tay-Sachs	1502	23271
***lyso*-Monosialoganglioside GM2 (*lyso*-GM2)**; synthesised shown in 3.1.1	-	-
N-omega-CD_3-Octadecanoyl monosialoganglioside GM2 (D_3GM2) (NH_4^+ salt); semisynthetic, human Tay-Sachs	2051	23202
Monosialoganglioside GM3 (NH_4^+ salt); natural, bovine buttermilk	1503	22074
***lyso*-Monosialoganglioside GM_3 (*lyso*-GM3)**; synthesised shown in 3.1.1	-	-
N-Hexadecanoyl-sulfatide; semisynthetic, bovine	1875	23056

The solvents, acetonitrile (ACN), methanol (MeOH) and water (H_2O), used for LC-MS/MS analysis were all HPLC grade from Merck or Sigma-Aldrich. The following mobile phases were used for LC-MS/MS experiments, mobile phase A (1.2% FA/0.38% ammonium acetate/H_2O), mobile phase B (1.2% FA/0.38% ammonium acetate/MeOH) and mobile phase C (0.1% FA/0.01% acetic acid/ACN). Other solvents used for sample preparation were all for analysis

from Merck. The cartridges used for solid phase extractions were OASIS MCX 1cc (30 mg) and Sep-Pack Vac 1cc (100 mg) from waters. For synthesis of *lyso*-GM2 and *lyso*-GM3 the enzyme sphingolipid ceramide N-deacylase, pseudomonas sp. (EC 3.5.1) from Calbiochem was used.

3.1.1. Synthesis of *lyso*-GM2 and *lyso*-GM3

As mentioned the substances of our interest were the *lyso*-forms of the sphingolipids. The two standards *lyso*-GM2 and *lyso*-GM3 couldn't be purchased, so each was synthesised from Monosialoganglioside GM2 or Monosialoganglioside GM3, which were available. The synthesis of *lyso*-GM2 and *lyso*-GM3 is an enzymatic synthesis, in which the N-linked fatty acid of ceramide is cleaved off, catalysed by sphingolipid ceramide N-deacylase.

This enzymatic synthesis was performed following the synthesis protocols described by Mills et al. (*38*) and Ito et al. (*39*). For the synthesis of *lyso*-GM2 and lyso-GM3 two different synthesis protocols were tried. In the first synthesis protocol 50 nmol of GM3 were incubated with 100 µl of sodium acetate buffer pH 5.0, with 5 mU sphingolipid ceramide N-deacylase and 0.8% fatty acid-free bovine serum albumin (BSA 1%, w/v). The reaction mixture was incubated for 16 h at 37°C. In the second synthesis protocol 50 nmol of each, GM2 and GM3, were incubated with 200 µl of sodium acetate buffer pH 5.0, with 10 mU sphingolipid ceramide N-deacylase and 0.8% fatty acid-free bovine serum albumin (BSA 1%, w/v). The reaction mixtures were incubated for 42 h at 37°C and 300 rpm.

After the incubation, *lyso*-GM2 and *lyso*-GM3 were isolated and purified with solid phase extraction (SPE), using C 18 cartridges, Sep-Pack Vac 1cc (100 mg) from waters. Therefore two different purification methods were tested. The first method (Method 1) was performed following the purification protocol described by Fauler et al. (*40*) and was tested for the purification of *lyso*-GM3 synthesised with the first protocol. The second method (Method 2) was performed following the purification protocol by Mills et al. (*38*) and was tested for the purification of *lyso*-GM2 and *lyso*-GM3 synthesised with the second protocol. The two different purification protocols (Method 1 and Method 2) are shown in table 2.

Table 2: Purification protocols for *lyso*-GM2 and *lyso*-GM3 with solid phase extraction

	Method 1	Method 2
1. Condition	1 ml methanol	1 ml methanol
2. Equilibrate	1ml H_2O	1ml H_2O
3. Lode cartridges with sample		
4. Wash	1ml H_2O 1 ml 60% aqueous methanol 500 µl 70% aqueous methanol 500 µl hexane 1 ml hexane:ethyl acetate (1:1, v/v) 1 ml toluol	1ml H_2O 1 ml 60% methanol 1 ml 70% methanol
5. Elute	3 ml acetone:methanol (9:1, v/v)	2 ml methanol 2 ml chloroform:methanol (2:1, v/v)

Each fraction of wash and elution of both methods were collected separately and were analysed with orbitrap by direct injection. Therefore an adequate amount of each fraction was evaporate to dryness under nitrogen and then resolved in mobile phase B. The fractions with the highest yield of *lyso*-GM2 and *lyso*-GM3 were evaporated to dryness under nitrogen at 37°C. The dried fractions were resolved in 1 ml of chloroform:methanol (2:1, v/v). The concentrations of the resolved *lyso*-GM2 and *lyso*-GM3 fractions were determined over the internal standard (D_3GM2) and were used for further experiment.

3.2. Devices

The LC-MS/MS experiments were done with high performance liquid chromatography (HPLC) coupled with a high-resolution mass spectrometer (Q Exactive orbitrap). The separate devices are listed below in table 3. For the LC-MS/MS method development different columns were used, which are also shown in table 3.

Table 3: LC-MS/MS devices used for analysis

LC-MS/MS	
Stack (cooled)	Thermo Fisher Scientific
Autosampler	Accela Open AS, Thermo Fisher Scientific
Column	1. Thermo Scientific Hypersil GOLD C 18 1.9 µm (50 x 2.1 mm) 2. Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm) 3. Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) 4. Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) 5. Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm) 6. Thermo Scientific Hypercarb 3 µm (100 x 3 mm) 7. Thermo Scientific Hypersil GOLD CN 3 µm (150 x 4 mm)
Oven with column switching unit	MistraSwitch, Maylab, Vienna, Austria
UHPLC-pump	1250 Accela, Thermo Fisher Scientific
Mass spectrometer	Q Exactive hybrid quatrupole-orbitrap mass spectrometer, Thermo Fisher Scientific
Software	Thermo Xcalibur 2.2 SP1.48, Thermo Fisher Scientific

3.3. ESI settings and structure analysis of standards

First of all the ESI settings were adjusted to generate optimal detection of the substances of interest. These adjusted ESI settings were saved as tune files. For the analysis of the substances of interest two tune files with different parameters were necessary. One tune file for the substances psychosine, glucosylsphingosine, glucosylsphingosine (plant), *lyso*-globotriaosylsphingosine, *lyso*-lactosylceramide and *lyso*-sphingomyelin which are detected as positive ions $[M+H]^+$ in ESI positive-ion-mode and another tune file for the gangliosides, lyso-gangliosides (GM1, GM2, GM3) and *lyso*-sulfatide which are detected as negative ions $[M-H]^-$ in ESI negative-ion-mode. The ESI settings of these two generated tune files are shown in table 3 and 4.

Table 4: ESI tune file for *lyso*-glycosphingolipids and *lyso*-sphingomyelin measured in positive-ion-mode

Tune file parameters for positive-ion-mode	
File name	GB3_lyso_tune
Polarity	positive
Sheath gas flow rate	60
Aux gas flow rate	20
Sweep gas flow rate	0
Spray voltage (|kV|)	5.00
Spray current (µA)	-
Capillary temp (°C)	350
S-lens RF level	50
Aux gas heater temp (°C)	300

Table 5: ESI tune file for gangliosides and sulfatide measured in negative-ion-mode

Tune file parameters for negative-ion-mode	
File name	Ganglioside_neg_tune
Polarity	negative
Sheath gas flow rate	60
Aux gas flow rate	20
Sweep gas flow rate	0
Spray voltage (|kV|)	4.50
Spray current (µA)	-
Capillary temp (°C)	350
S-lens RF level	50
Aux gas heater temp (°C)	300

After the generation of ESI tune files, all standards and internal standards were analysed with the orbitrap by direct injection. Therefore standard solutions with 5 µg/ml in mobile phase A:mobile phase C (1:1; v/v), for substances analysed in positive-ion-mode and standard solutions with 500 ng/ml in mobile phase A:mobile phase C (1:1; v/v), for substances analysed in negative-ion-mode were used.

3.4. LC-MS/MS method development

An adequate LC-MS/MS method was needed to analyse the *lyso*-sphingolipids, accumulation products of the different lysosomal storage diseases. As mentioned the intention was to develop a single LC-MS/MS method in which all accumulation products of the different lysosomal storage diseases are covered. After some reflections it was clear that the accumulating substances are quite diverse in retention and ionisation behaviour and it is not possible to cover them all within a single method. Therefor the following three methods were developed to analyse the accumulating *lyso*-sphingolipids of the different lysosomal storage disorders.

All LC-MS/MS experiment were performed with high performance liquid chromatography (HPLC) coupled with a high-resolution mass spectrometer (Q Exactive orbitrap), listed in *3.2. Devices*. The MS/MS methods were all performed as targeted SIM experiments. Therefore the exact *m/z* of the substances and adducts are necessary to insert them in the inclusion list. The exact m/z were determined in *4.2. Structure analysis of standards and internal standards*.

3.4.1. Psychosine and Glucosylsphingosine

The *lyso*-glycosphingolipids, psychosine and glucosylsphingosine, are quite similar in their structure and mass. Therefor it was rather difficult to separate them with a chromatographic method and it is impossible to separate them over the *m/z*.

A variety of chromatographic methods with different columns and gradients were tested. The following columns Thermo Scientific Hypersil GOLD C 18 1.9 µm (50 x 2.1 mm), Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm), Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm), Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm) and Thermo Scientific Hypercarb 3 µm (100 x 3 mm) were tested for the separation of psychosine and glucosylsphingosine with various chromatographic gradients. All tested methods and chromatographic gradients are shown in appendix, *9.1. Psychosine and Glucosylsphingosine*. However the tested columns and gradients did not lead to the required separation.

Finally the separation of psychosine and glucosylsphingosine was achieved following the chromatographic separation described by Welford at al.(*21*) The LC-MS/MS settings are shown in the table 6 below. Instead of a 150 mm column as described by Welford at al., two 5 mm HILIC columns were used to achieve the separation. The chromatographic method with an

isocratic gradient is shown in table 7. For the detection of psychosine and glucosylsphingosine a targeted SIM experiment was performed.

Table 6: LC-MS/MS settings for chromatographic separation and detection of psychosine and glucosylsphingosine

Parameters	
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Flow rate	400 µl/min
Injection volume	10 µl
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Targeted SIM; positive; resolution: 70 000; AGC target: $5e^4$; maximum IT: 200 ms; MSX count: 4; Isolation window: 1.0 *m/z*; scan range: 400-820 *m/z*; Timed inclusion list 2.5-7 min: psychosine and glucosylsphingosine (*m/z* 462.34308/484.32502), glucosylsphingosine (plant) (*m/z* 460,32743/482.30937)

Table 7: Chromatographic isocratic gradient for the separation of psychosine and glucosylsphingosine

Time [min]	Mobile phase A [%]	Mobile phase B [%]	Mobile phase C [%]
0	7	7	86
8	7	7	86

3.4.2. *lyso*-Glycosphingolipids and *lyso*-Sphingomyelin

The *lyso*-glycosphingolipids (psychosine, glucosylsphingosine and *lyso*-Gb3) and lyso-sphingomyelin are similar in retention behaviour and ionisation, therefore it is possible to separate and analyse them within one LC-MS/MS method.

A variety of chromatographic methods with different columns and gradients were tested. The following columns, Thermo Scientific Hypersil GOLD C 18 1.9 µm (50 x 2.1 mm), Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm) and Thermo Scientific Hypersil GOLD CN 3 µm (150 x 4 mm) were tested for the separation of psychosine, glucosylsphingosine, *lyso*-Gb3, *lyso*-LC and SPC with various chromatographic gradients. All tested methods and chromatographic gradients are shown in appendix, *9.2. lyso-Glycosphingolipids and lyso-Sphingomyelin*. However the tested columns and gradients did not lead to the required separation.

Finally the separation and the detection were achieved with the LC-MS/MS settings given in table 8 and the chromatographic gradient, shown in table 9.

Table 8: LC-MS/MS settings for chromatographic separation and detection of *lyso*-glycosphingolipids and *lyso*-sphingomyelin

Parameters	
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Flow rate	500 µl/min
Injection volume	10 µl
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Targeted SIM; positive; resolution: 70 000; AGC target: $5e^4$; maximum IT: 200 ms; MSX count: 10; Isolation window: 1.0 *m/z*; scan range: 400-820 *m/z*; Timed inclusion list 4-10 min: psychosine and glucosylsphingosine (*m/z* 462.34308/484.32502), SPC (d16:1) (*m/z* 437.31444/459.29638), SPC (d18:1) (*m/z* 465.34574/487.32768), *lyso*-Gb3 (*m/z* 786.44873/808.43067), *lyso*-LC (*m/z* 624.39590/646.37785)

Table 9: Chromatographic gradient for the separation of *lyso*-glycosphingolipids and *lyso*-sphingomyelin

Time [min]	Mobile phase A [%]	Mobile phase C [%]
0	40	60
12	0	100
14	0	100
15	40	60
20	40	60

3.4.3. Gangliosides and *lyso*-Sulfatide

The gangliosides *lyso*-GM1, GM2, GM3 and *lyso*-Sulfatide form negative charged $[M-H]^-$ molecules, so they are all analysed with ESI in negative-ion-mode and because of their structure similarity it was possible to separate them in a single LC-MS/MS method.

There already was an existing method established by Thomas Kaiser at LKH Graz. The separation of the gangliosides was achieved, in the existing method, with a C18 column. The existing chromatographic gradient and the LC-MS/MS method were modified for the separation with the Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm) column instead of the C18

column. All tested methods and chromatographic gradients are shown in appendix, *9.3. Gangliosides and lyso-Sulfatide*. The separation and the detection of gangliosides and *lyso*-sulfatide was achieved with the LC-MS/MS settings given in table 10 and the chromatographic gradient shown in table 11.

Table 10: LC-MS/MS settings for chromatographic separation and detection of gangliosides and *lyso*-sulfatide

Parameters	
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Flow rate	600 µl/min
Injection volume	10 µl
Ion source	ESI (electrospray ionization)
Ionization settings	Ganglioside_neg_tune (see 3.3.)
MS/MS	Targeted SIM; negative; resolution: 70 000; AGC target: $5e^4$; maximum IT: 200 ms; MSX count: 6; isolation window: 1.0 *m/z*; scan range: 500-1500 *m/z*; timed inclusion list 3-10 min: *lyso*-Sulfatide (*m/z* 540.28424), *lyso*-GM1 (*m/z* 1278.60786/ 1306.63916); 10-15 min: GM2 (*m/z* 1382.81601/1410.84731), D_3GM2 (*m/z* 1385.83484/ 1413.86614), GM3 (*m/z* 1151.70533/1179.73663); 15-20 min: GM3 (*m/z* 1207.7679/ 1221.78358/1235.79923/1249.81488/1263.83053/1277.8473)

Table 11: Chromatographic gradient for the separation of gangliosides and *lyso*-sulfatide

Time [min]	**Mobile phase A [%]**	**Mobile phase B [%]**
0	20	80
10	0	100
20	0	100
21	20	80
30	20	80

3.4.4. *lyso*-Gangliosides and *lyso*-Sulfatide

The *lyso*-gangliosides and *lyso*-sulfatide have a similar retention behaviour as the gangliosides. Therefore the chromatographic separation of the *lyso*-gangliosides and *lyso*-sulfatide was achieved with the same chromatographic gradient as for gangliosides, shown in table 11. The LC-MS/MS settings are shown in table 12 and only differ in the MS/MS settings in comparison to the gangliosides.

Table 12: LC-MS/MS settings for the chromatographic separation and the detection of *lyso*-gangliosides and *lyso*-sulfatide

Parameters	
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Flow rate	600 µl/min
Injection volume	10 µl
Ion source	ESI (electrospray ionization)
Ionization settings	Ganglioside_neg_tune (see 3.3.)
MS/MS	Targeted SIM; negative; resolution: 70 000; AGC target: $5e^4$; maximum IT: 200 ms; MSX count: 8; isolation window: 1.0 *m/z*; scan range: 500-1500 *m/z*; timed inclusion list 3-20 min: *lyso*-Sulfatide (*m/z* 540.28424), *lyso*-GM1 (*m/z* 1278.60786/ 1306.63916), D_3GM2 (*m/z* 1385.83484/1413.86614), *lyso*-GM2 (*m/z* 1116.55504/ 1144.58634), *lyso*-GM3 (*m/z* 913.47567)

3.5. Sample Preparation

The sample preparation was performed following the protocol described by Boutin et al. (*41*). For the sample preparation 200 µl of well-mixed EDTA-plasma or serum were used. To the 200 µl sample aliquots, 100 µl of each internal standard (100 ng/ml in methanol) was added. For the protein precipitation and acidification 400 µl methanol and 500 µl H_3PO_4 (2% in H_2O) were added. The well-mixed sample mixtures were centrifuged at 3000 rpm for 10 min at RT and the supernatants were transferred to the preconditioned cartridges OASIS MCX 1cc (30 mg) from waters. The cartridges were preconditioned with 1200 µl of methanol and 1200 µl of H_3PO_4 (2% in H_2O). After loading the samples, the cartridges were first washed with 1200 µl of 2% formic acid (FA) in H_2O and then with 1200 µl of 0.2% FA in methanol. The *lyso*-glycosphingolipids were eluted five times with 600 µl of 2% ammonia in methanol. The eluates were evaporated to dryness at 37°C under nitrogen. The dried samples were resolved in 100 µl of mobile phase A:mobile phase C (1:1, v/v) and then transferred to vials for LC-MS analysis.

3.6. Calibration, quantification and method validation

The calibration, quantification and method validation was only done for the substances, *lyso*-Glycosphingolipids and *lyso*-Sphingomyelin, because for the gangliosides, *lyso*-gangliosides and *lyso*-sulfatide no adequate standard was available. Furthermore normal values in plasma samples and the concentrations in plasma samples of patients with a lysosomal storage disorder were determined.

3.6.1. Psychosine and Glucosylsphingosine

A separate calibration was done for psychosine and glucosylsphingosine with the following standard concentrations 100, 50, 25, 12.5, 6.25, 3.125 ng/ml. To 50 µl of each standard 100 µl of internal standard, GLY plant (100 ng/ml), were added. Instead of 200 µl EDTA-plasma or serum 150 µl MeOH was added to get the same volume as for the plasma samples. These samples were further prepared as described in *3.5. Sample preparation*.

In the method validation, limit of quantification (LOQ) and limit of detection (LOD), were determined following the protocol described by Boutin et al.(*41*) Therefore the area ratio of PSY/IST and GLY/IST of standard 5 was determined (n=5). The LOD was calculated by three times of the standard deviation of the area ratio, divided by the slope of the calibration curve. Whereas the LOQ is calculated by ten times of the standard deviation of the area ratio. In addition normal values of psychosine and glucosylsphingosine were determined by analysing plasma samples of healthy controls (n=10).

3.6.2. *lyso*-Glycosphingolipids and *lyso*-Sphingomyelin

The calibration was done for psychosine and glucosylsphingosine (*lyso*-HexosylCer) with the following standard concentrations 100, 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195 ng/ml. For *lyso*-globotriaosylsphingosine the calibration was done with the following standard concentrations 50, 25, 12.5, 6.25, 3.125, 1.563, 0.781, 0.391, 0.195, 0.098 ng/ml and for lyso-sphingomyelin the following standard concentrations 69.552, 34.776, 17.388, 8.694, 4.347, 2.174 ng/ml were used. To 50 µl of each standard 100 µl of internal standard, *lyso*-lactosylceramide (100 ng/ml), were added. Instead of 200 µl EDTA-plasma or serum 150 µl MeOH were added to get the same volume as for the plasma samples. These samples were further prepared as described in *3.5. Sample preparation*.

The method validation for each substance was done as described in *3.6.1.* for psychosine and glucosylsphingosine. Furthermore the reproducibility of the sample preparation and analysis was determined by analysing a plasma sample of a healthy control (n=6). In addition normal values of *lyso*-HexosylCer, *lyso*-Gb3, SPC were determined by analysing plasma samples of healthy controls (n=10).

4. Results

4.1. Analysis and quantification of synthesised *lyso*-GM2 and *lyso*-GM3

First of all, as described in chapter *3.1.1.*, all eluted fractions were analysed with orbitrap by direct injection. In figure 8 and 9 the spectra of the fractions with the highest peak intensities of *lyso*-GM2 and *lyso*-GM3 are shown. With direct injection not only the fractions with the highest concentrations of *lyso*-GM2 and *lyso*-GM3, but also the different molecule species contained in the standards and their related monoisotopic ion masses $[M-H]^-$ were determined. In the spectra the calculated monoisotopic ion mass is given in bracket beside the identified peak.

In figure 8 below the spectrum of the *lyso*-GM2 standard with the highest yield, synthesised with the second synthesis protocol and 1. eluate of method 2, is shown. In the obtained spectrum two peaks were identified as different species of *lyso*-GM2. The first species contained in *lyso*-GM2 standard is the *lyso*-GM2 with a sphingosine (d18:1) backbone, which corresponds to the peak at *m/z* 1116.5550. The other peak at *m/z* 1144.5863 corresponds to *lyso*-GM2 with a sphingoid base (d20:1) backbone.

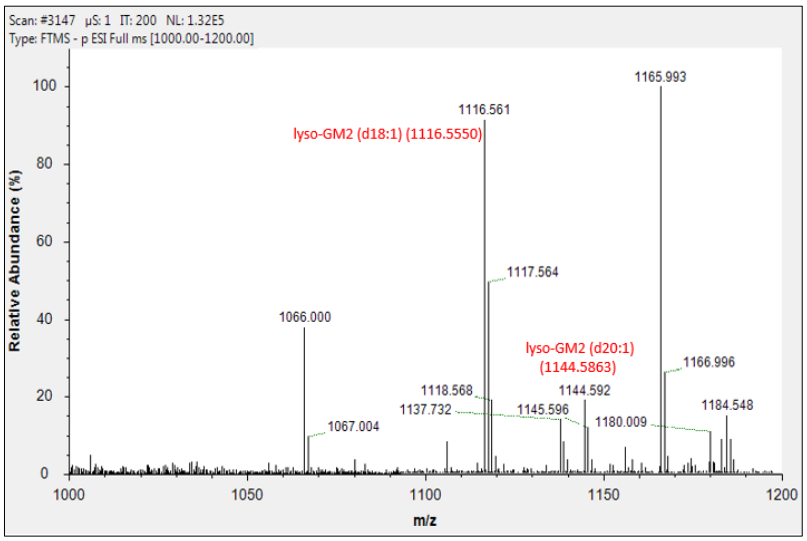

Figure 8: Spectrum of lyso-GM2 with direct injection

In figure 9 below the spectrum of the *lyso*-GM3 standard with the highest yield, synthesised with the first synthesis protocol and eluate of method 1, is shown. The obtained spectrum only shows a single peak at *m/z* 913.4757, which corresponds to *lyso*-GM3 with a sphingosine (d18:1) backbone.

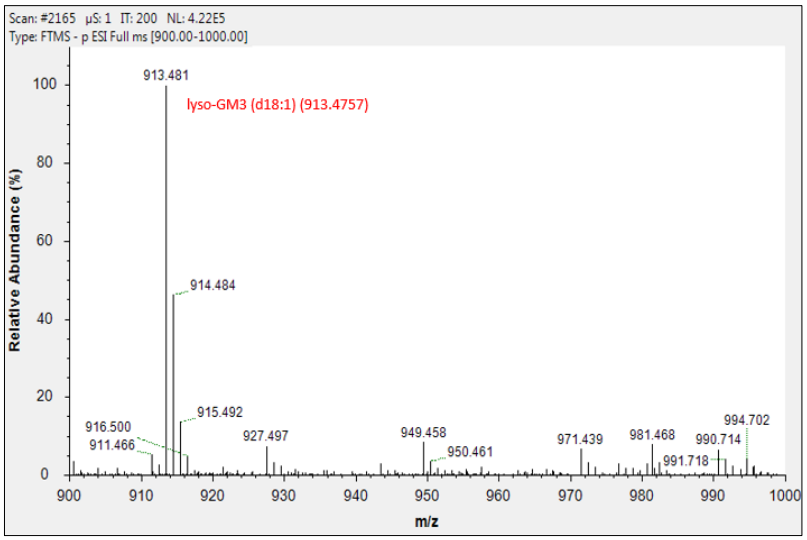

Figure 9: Spectrum of *lyso*-GM3 with direct injection

The concentrations of the synthesised *lyso*-GM2 and *lyso*-GM3 standards were determined over the internal standard D_3GM2 as described in *3.1.1.*. The results of the quantification are shown in table 13.

Table 13: Concentration and yield of lyso-GM2 and lyso-GM3 standards

Standard	Concentration [ng/ml]	Yield [%]
lyso-GM2	750	1,50
lyso-GM3	1280	2,56

The yield of the enzymatic syntheses for the products *lyso*-GM2 and *lyso*-GM3 is 1-3 % which is quite low. For the further analysis, the method development, the quality of the standards *lyso*-GM2 and *lyso*-GM3 were adequate.

4.2. Structure analysis of standards and internal standards

As described in *3.3. ESI settings and structure analysis of standards*, the purchased standards and internal standards were analysed with orbitrap by direct injection. It was done to identify the structure and the different species contained in the standards, because they are all natural or semisynthetic from different organisms as bovine, bovine buttermilk, porcine and human. Therefore it is possible that the standards contain various sphingoid base backbones and different *N*-linked fatty acid residues. Therefore it is necessary to determine the different species contained in the standards and the formed adducts, to perform targeted SIM experiments. In the spectra the calculated monoisotopic ion mass is given in bracket beside the identified peak.

Psychosine and Glucosylsphingosine

In figure 10 the spectra of the psychosine and the glucosylsphingosine standards are shown. The psychosine standard is semisynthetic from bovine and the glucosylsphingosine standard is semisynthetic from bovine buttermilk. There are prominent molecular ion peaks $[M-H]^+$ at *m/z* 462.3431 in both spectra which corresponds to psychosine and glucosylsphingosine with a sphingosine (d18:1) backbone. The peaks at *m/z* 484.3250 are the associated Na-adducts. As it can be seen in the spectrum of psychosine there is just psychosine with a sphingosine (d18:1) backbone contained in the standard. Whereas in the spectrum of glucosylsphingosine it can be seen that there is also a little of the glucosylsphingosine (d16:1) species contained.

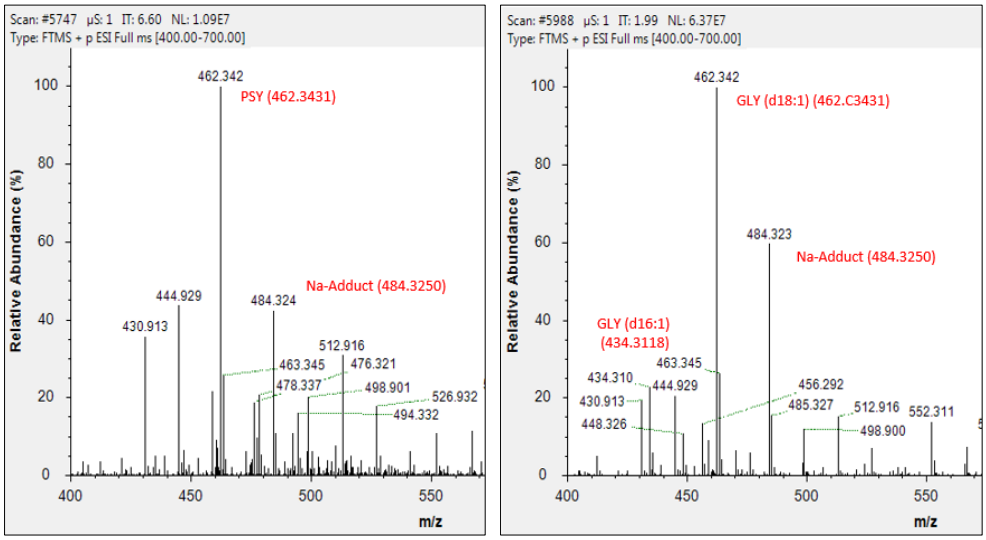

Figure 10: Spectra of psychosine (left) and glucosylsphingosine (right) standard

lyso-Globotriaosylsphingosine

In figure 11 the spectrum of the *lyso*-Globotriaosylsphingosine standard, semisynthetic from porcine RBC, is shown. There is a prominent molecular ion peak $[M-H]^+$ at *m/z* 786.4487 which corresponds to *lyso*-Gb3 with a sphingosine (d18:1) backbone. The peak at *m/z* 808.4307 is the associated Na-adduct. There is no other species than the sphingosine (d18:1) contained in the standard.

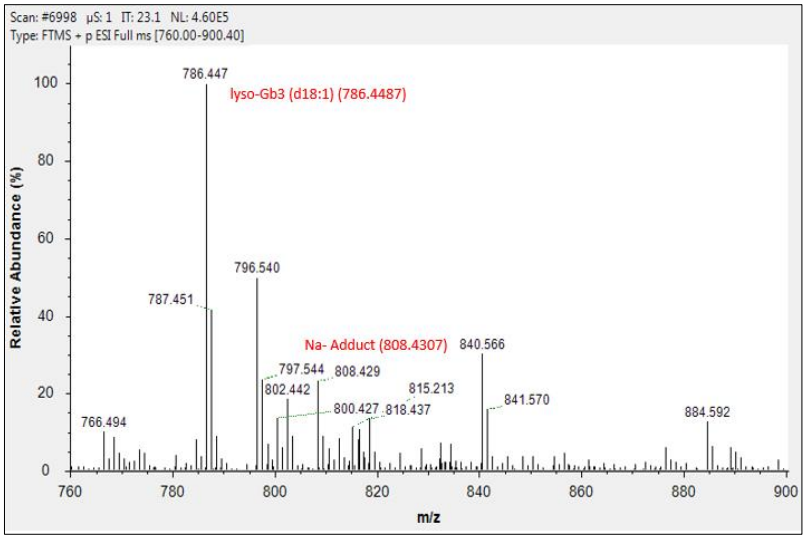

Figure 11: Spectrum of the *lyso*-Globotriaosylsphingosine standard

lyso-Sphingomyelin

In figure 12 the spectrum of the *lyso*-Sphingomyelin standard, semisynthetic from bovine buttermilk, is shown. There is a prominent molecular ion peak $[M-H]^+$ at *m/z* 465.3457 which refers to SPC with a sphingosine (18:1) backbone. The peak at *m/z* 487.3277 is the associated Na-adduct. Almost half of the standard consists of the second species with the sphingoid base (d16:1) backbone and the associated Na-adduct.

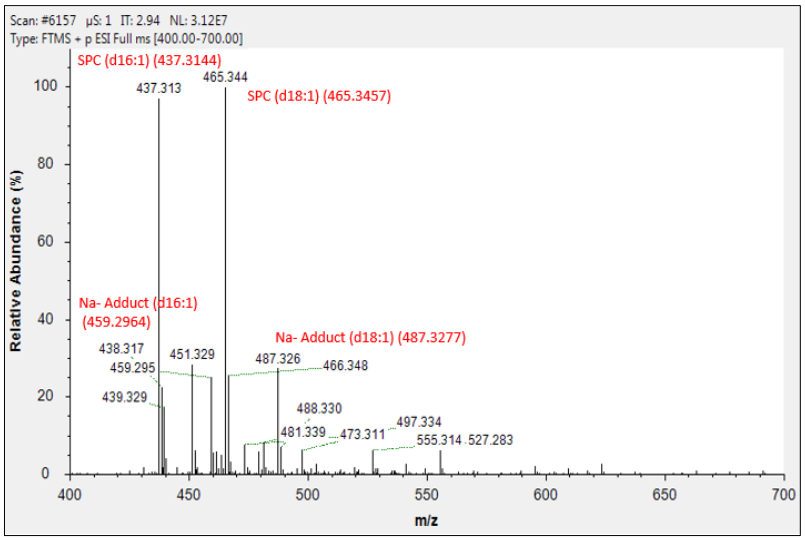

Figure 12: Spectrum of the lyso-sphingomyelin standard

lyso-GM1

In figure 13 the spectrum of the *lyso*-GM1 standard, semisynthetic from bovine, is shown. There is a prominent molecular ion peak $[M-H]^-$ at *m/z* 1278.6079 which corresponds to *lyso*-GM1 with sphingosine (d18:1) backbone. There is a second very intense peak in the spectrum at *m/z* 1306.6392 which corresponds to *lyso*-GM1 with a sphingoid base (d20:1) backbone. Furthermore there are two peaks with an equal distance of m/z 67.9874 to the two molecular ion peaks. These are probably sodium formate clusters of the two *lyso*-GM1 species.

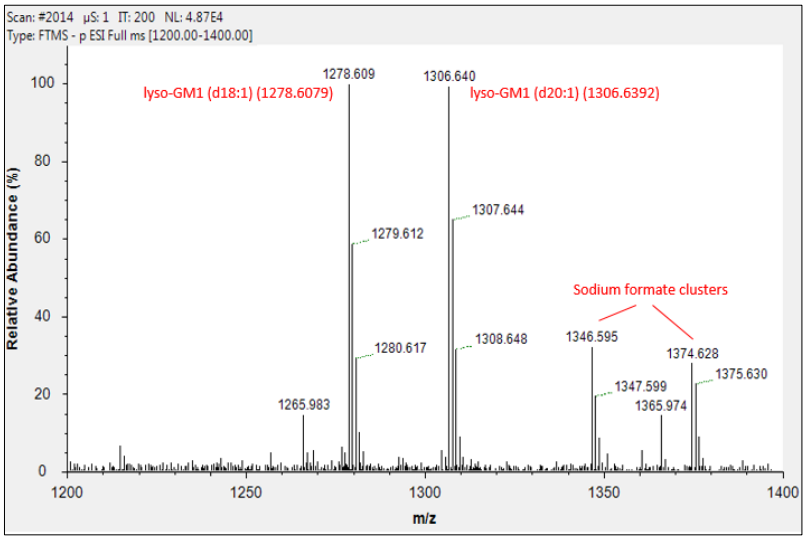

Figure 13: Spectrum of the *lyso*-GM1 standard

GM2

In figure 14 the spectrum of the GM2 standard, natural from human Tay-Sachs, is shown. There is a prominent molecular ion peak $[M-H]^-$ at *m/z* 1382.8160 which corresponds to GM2 (d18:1/18:0). The second peak at *m/z* 1410.8473 probably corresponds to GM2 (d20:1/18:0), but it is also possible that the GM2 (d18:1/20:0) is the corresponding substance according to the *m/z*. In *4.1.*, figure 8, the spectrum of *lyso*-GM2 is shown, there are two peaks detected one corresponds to the *lyso*-GM2 (d18:1) and the second one to the *lyso*-GM2 (d20:1). Hence the peak at *m/z* 1410.8473 corresponds to GM2 (d20:1/18:0), because with sphingolipid ceramide N-deacylase the N-linkage is cleaved and the fatty acids are split off. The *lyso*-glycosphingolipids with different sphingosine backbones result.

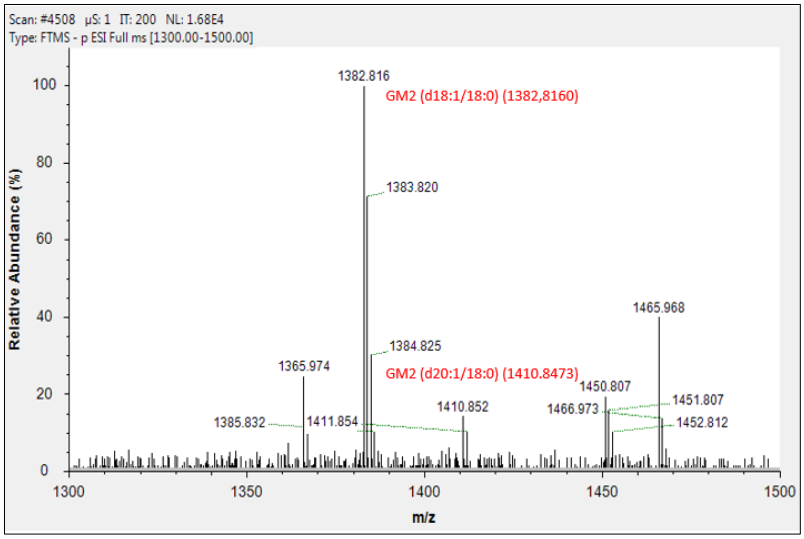

Figure 14: Spectrum of the GM2 standard

D_3GM2

In figure 15 the spectrum of the D_3GM2 standard, semisynthetic from human Tay-Sachs, is shown. There is a prominent molecular ion peak $[M-H]^-$ at *m/z* 1385.8348 which corresponds to D_3GM2 (d18:1/18:0). There is no other noticeable species or adduct detected in the spectrum.

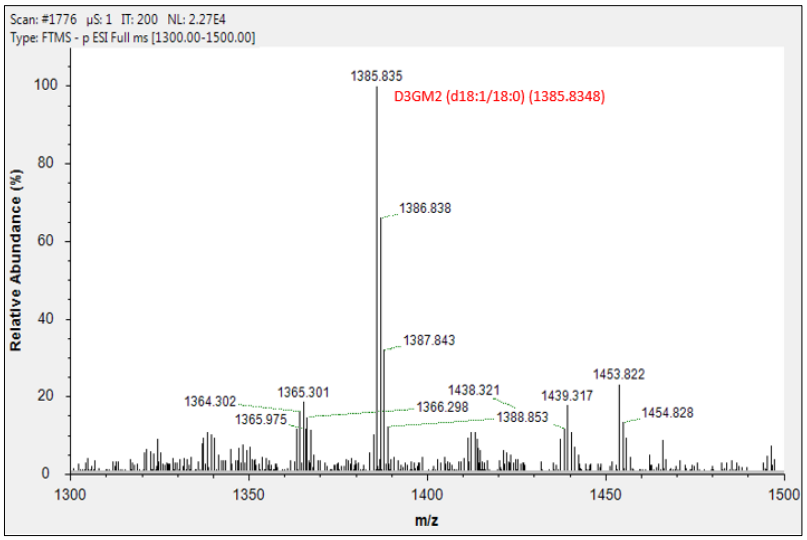

Figure 15: Spectrum of the D_3GM2 internal standard

GM3

In figure 16 the spectrum of the GM3 standard, natural from bovine buttermilk, is shown. There are a few prominent molecular ion peaks $[M-H]^-$ in the spectrum. The first peak at *m/z* 1151.7053 corresponds to GM3 (d18:1/16:0). The next one at *m/z* 1221.7836 corresponds to GM3 (d18:1/21:0). The following peaks have a distance of *m/z* 14.0157 to their previous peak which corresponds to an addition of -[CH_2]- from one peak to the other. As it can be seen in the spectrum there are the following GM3 species contained: GM3 (d18:1/18:22), GM3 (d18:1/18:23), GM3 (d18:1/18:24).

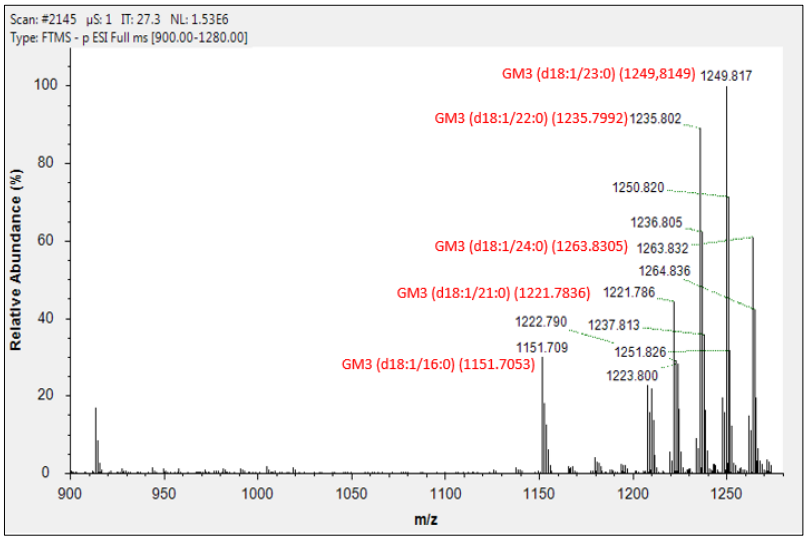

Figure 16: Spectrum of the GM3 standard

lyso-Sulfatide

In figure 17 the spectrum of the *lyso*-sulfatide standard, semisynthetic from bovine, is shown. There is a prominent molecular ion peak $[M-H]^-$ at *m/z* 540.2842 which corresponds to *lyso*-sulfatide with a sphingosine (d18:1) backbone. There is no other noticeable species or adduct detected in the spectrum.

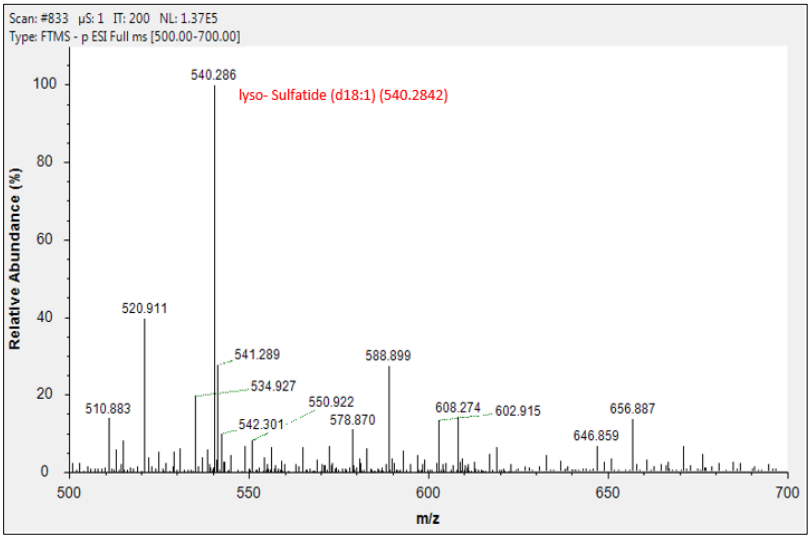

Figure 17: Spectrum of the *lyso*-Sulfatide standard

4.3. LC-MS/MS method development

4.3.1. Psychosine and Glucosylsphingosine

The lyso-glycosphingolipids, psychosine and glucosylsphingosine, only differ in the carbohydrate group linked to position 1. In psychosine a galactose and glucosylsphingosine a glucose is linked to the sphingoid base, so the only difference is the steric OH-group in the carbohydrate at position 4. Therefore it is not possible to distinguish these two substances according to their *m/z*. The only differentiation of the two substances is the chromatographic separation.

In figure 18 the achieved chromatographic separation of psychosine and glucosylsphingosine, with the developed method in *3.4.1. Psychosine and Glucosylsphingosine*, is shown. The two peaks are not base line separated, but it is possible to distinguish between psychosine and glucosylsphingosine, because there is a little shift in the retention time (RT).

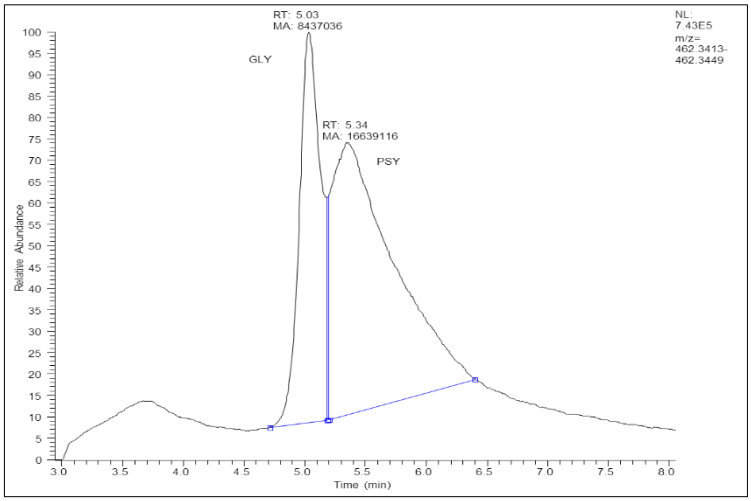

Figure 18: Chromatographic separation of psychosine and glucosylsphingosine

In patients never both substances are elevated, there is either psychosine or glucosylsphingosine accumulated. In Gaucher patients the glucosylsphingosine level is elevated and in Krabbe patients the accumulation product is psychosine. In figure 19 the chromatograms of glucosylsphingosine and psychosine in comparison to the internal standard (glucosylsphingosine plant) are shown. In comparison to the internal standard, the glucosylsphingosine peak has almost the same RT of 4.77 min, whereas the psychosine peak is slightly shifted backwards to a RT of 4.90 min.

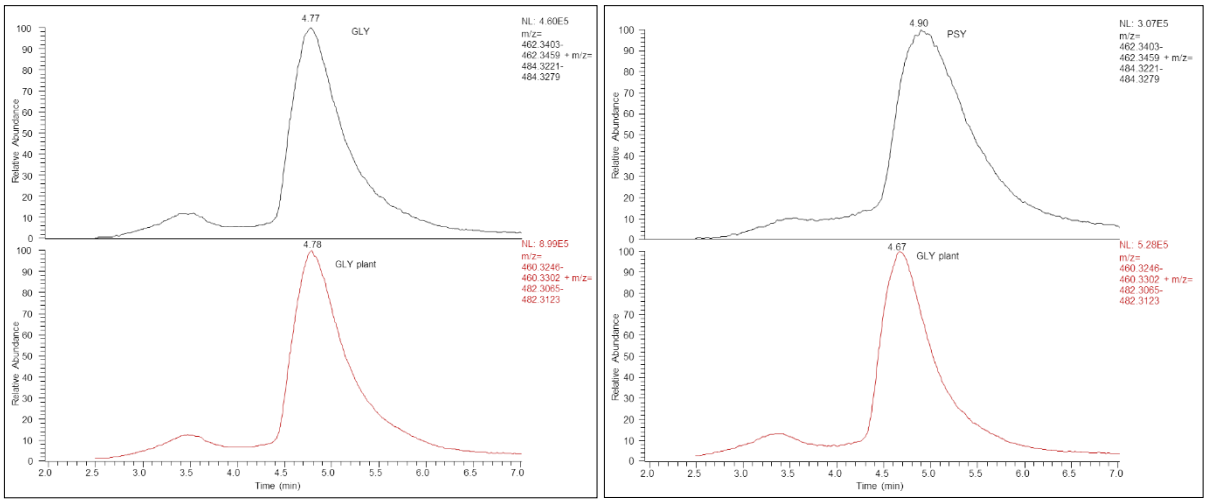

Figure 19: Chromatograms of glucosylsphingosine and psychosine in comparison to glucosylsphingosine plant (IST). The chromatogram on the left is showing glucosylsphingosine having near the same RT as the IST. The right one is showing psychosine in comparison to the IST.

4.3.2. *lyso*-Glycosphingolipid and *lyso*-Sphingomyelin

The achieved chromatographic separation of psychosine, glucosylsphingosine, *lyso*-Gb3 and *lyso*-sphingomyelin, with the developed method described in *3.4.2.*, is shown in figure 20. In the first line the total ion current chromatogram (TIC) is shown. The second line in red shows a peak at a RT of 6.79 min which corresponds to *lyso*-HexosylCer (psychosine and glucosylsphingosine). With this method it is not possible to separate psychosine and glucosylsphingosine, therefore the method, in *3.4.1. Psychosine and Glucosylsphingosine*, has been established. The third line in green shows a quite broad double peak at a RT of 5.93/6.27 min which corresponds to *lyso*-sphingomyelin. The fourth line in blue shows a single peak at a RT of 4.84 min which corresponds to *lyso*-Gb3. The fifth line in yellow shows a double peak at a RT of 5.32/5.59 min which corresponds to the internal standard *lyso*-lactosylceramide.

The chromatogram, in figure 20, shows an adequate separation of *lyso*-HexosylCer, *lyso*-Gb3, *lyso*-sphingomyelin and internal standard, only psychosine and glucosylsphingosine are not separated. If *lyso*-HexosylCer is elevated in a plasma sample within this separation method, the method described in *3.4.1. Psychosine and Glucosylsphingosine*, should be used, to identify whether it is a Gaucher or Krabbe patient.

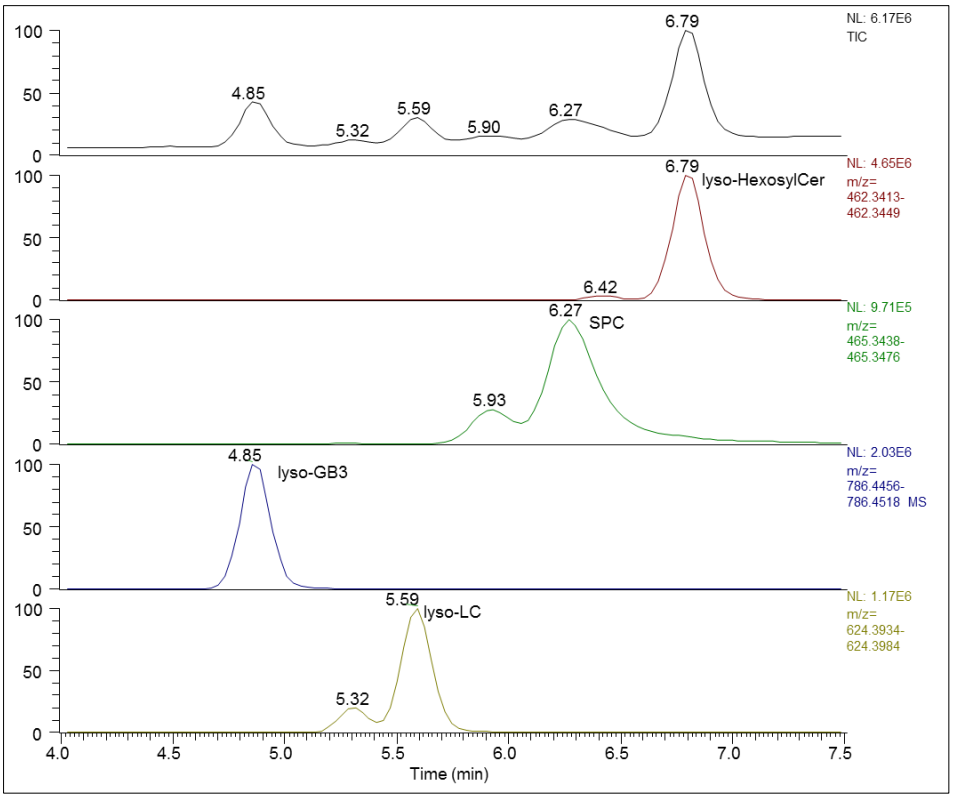

Figure 20: Chromatographic separation of *lyso*-glycosphingolipids and *lyso*-sphingomyelin

4.3.3. Gangliosides and *lyso*-Sulfatide

The achieved chromatographic separation of *lyso*-GM1, GM2, *lyso*-sulfatide and the internal standard (D_3GM2), with the developed method described in *3.4.3. Gangliosides and lyso-Sulfatide*, is shown in figure 21. In the first line the total ion current chromatogram (TIC) is shown. In the second and third line the two species of *lyso*-GM1 are shown. The second line in red shows a peak at a RT of 5.96 min which corresponds to the *lyso*-GM1 (d18:1). The third line in green shows a peak at a RT of 7.82 min which corresponds to *lyso*-GM1 (d20:1). The fourth line in blue shows a peak at a RT of 14.08 min which corresponds to GM2 (d18:1/18:0). The GM2 (d20:1/18:0) which was identified in the structure analysis cannot be found in the chromatogram. The fifth line in yellow shows a peak at a RT of 6.64 min which corresponds to the *lyso*-sulfatide. The last line in purple shows a peak at a RT of 14.06 min which corresponds to the internal standard D_3GM2 (d18:1/18:0).

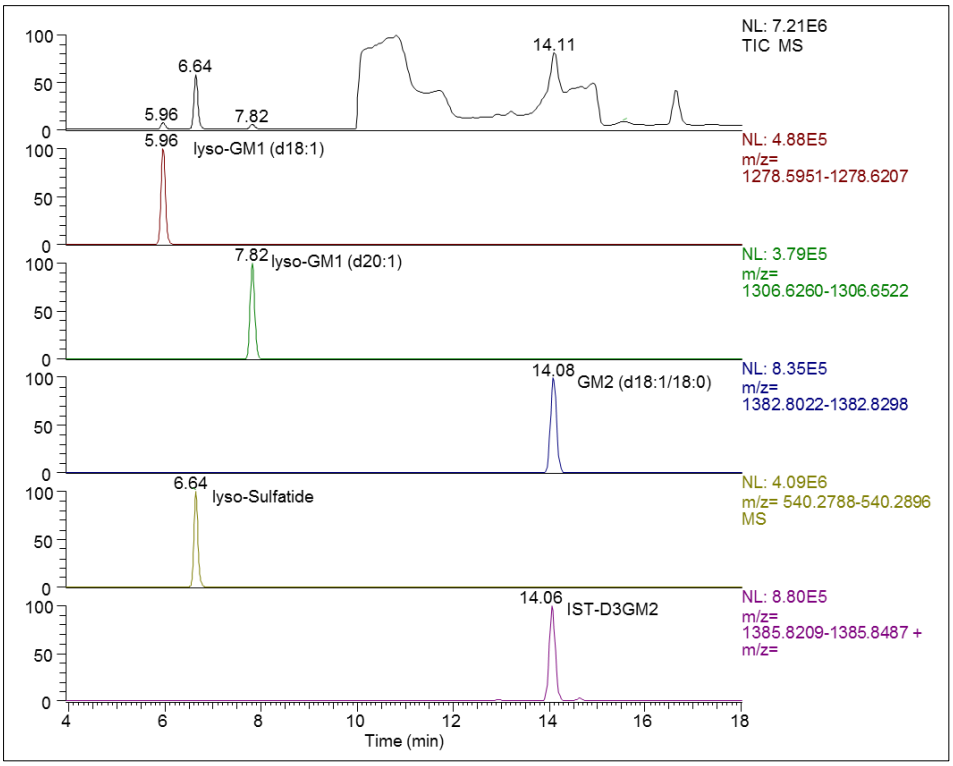

Figure 21: Chromatogram of ganglioside and sulfatide

The achieved chromatographic separation of the GM3 species and the internal standard (D_3GM2), with the developed method described in *3.4.3.*, are shown in figure 22 and 23. In the first line the total ion current chromatogram (TIC) is shown. The second line in red shows a double peak at a RT of 12.92 min which corresponds to GM3 (d18:1/16:0). The next line in green shows a peak at a RT of 14.03 min which corresponds to GM3 (d18:1/18:0). The fourth line in blue shows a peak at a RT of 15.04 which corresponds to GM3 (d18:1/20:0). The following line in yellow shows a peak at a RT of 15.62 min which corresponds to GM3 (d18:1/21:0). The last line shows a peak at a RT of 14.06 min which corresponds to the internal standard D_3GM2 (d18:1/18:0). In figure 23 the first line in black shows also the TIC. The second line in red shows three peaks at a RT of 16.00/16.26/16.59 min which corresponds to GM3 (d18:1/22:0). The next line in green shows a double peak at a RT of 16.69/17.35 which corresponds to GM3 (d18:1/23:0). The following line in blue shows a double peak at a RT of 17.45/18.11 min which corresponds to GM3 (d18:1/24:0). The line in yellow shows a peak at a RT of 18.23 min which corresponds to GM3 (d18:1/25:0). The last line shows the internal standard D_3GM2 (d18:1/18:0) again.

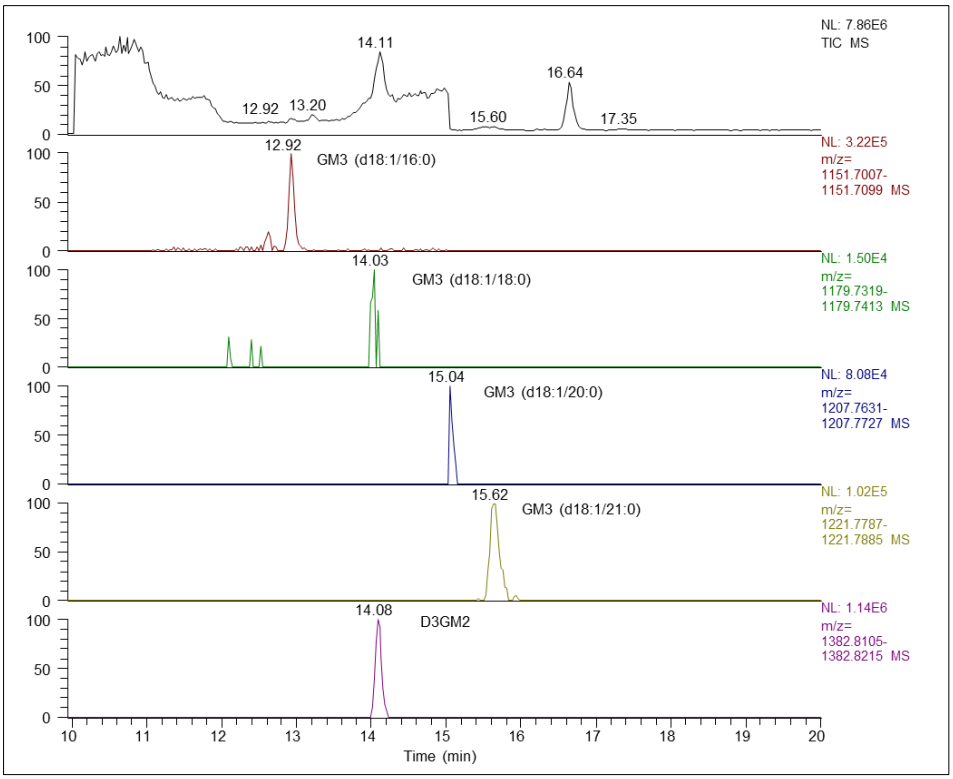

Figure 22: Chromatogram of the different species of ganglioside GM3

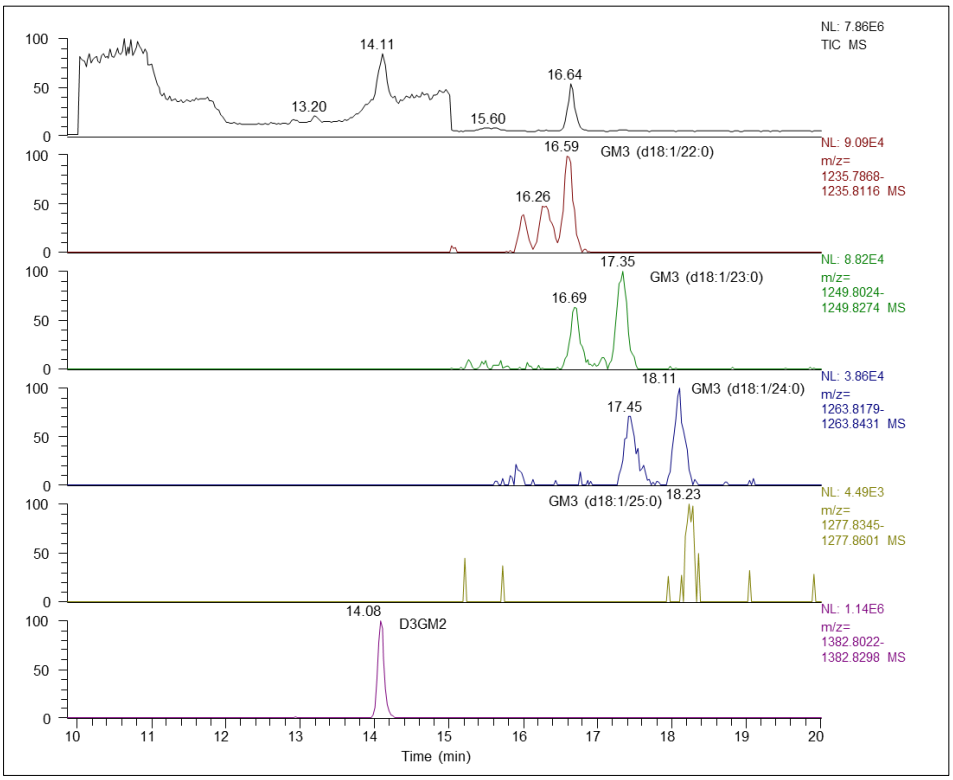

Figure 23: Chromatogram of the different species of ganglioside GM3

4.3.4. *lyso*-Gangliosides and *lyso*-Sulfatide

The achieved chromatographic separation of *lyso*-gangliosides, *lyso*-sulfatide and the internal standard (D_3GM2), with the developed method described in *3.4.4.*, is shown in figure 24. In the first line the total ion current chromatogram (TIC) is shown. In the second and third line the two species of *lyso*-GM1 are shown. The second line in red shows a peak at a RT of 5.98 min which corresponds to the *lyso*-GM1 with a sphingosine (d18:1) backbone. The third line in green shows a peak at a RT of 7.87 min which corresponds to *lyso*-GM1 (d20:1). The fourth line in blue shows a peak at a RT of 6.14 min which corresponds to *lyso*-GM2 with a sphingosine (d18:1) backbone. The fifth line in yellow shows a peak at a RT of 8.06 min which corresponds to *lyso*-GM2 (d20:1). The sixed line in purple shows a double peak at a RT of 5.85/6.20 min which corresponds to *lyso*-GM3 (d18:1). The seventh line in cyan shows a peak at a RT of 6.68 min which corresponds to the *lyso*-sulfatide (d18:1). The last line in grey shows a peak at a RT of 14.09 min which corresponds to the internal standard D_3GM2 (d18:1/18:0).

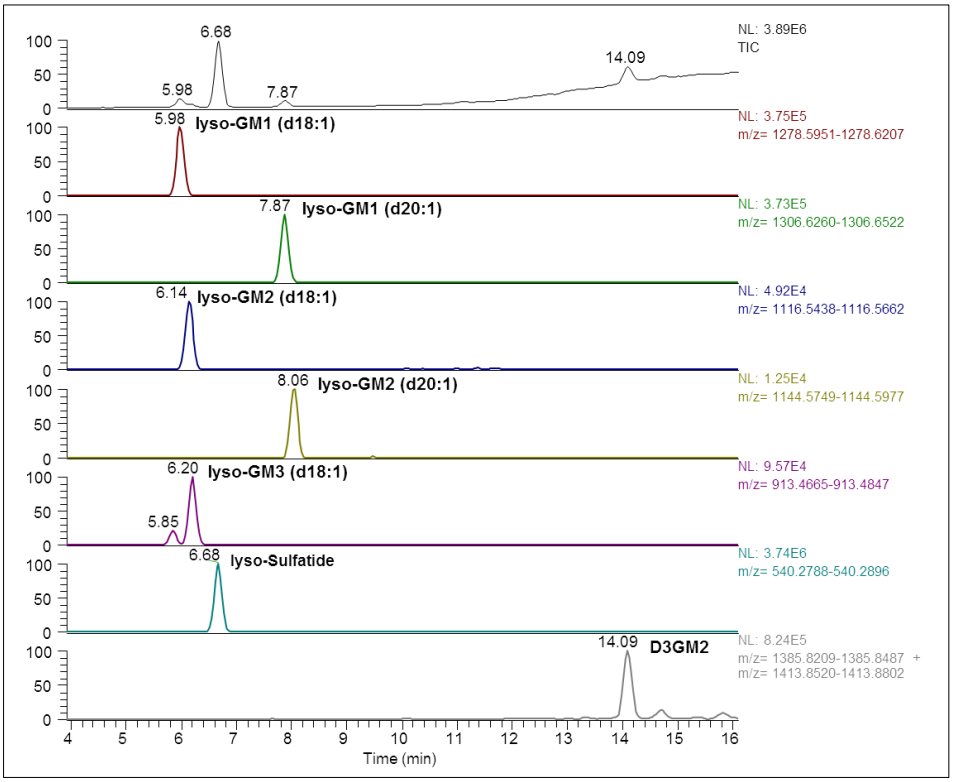

Figure 24: Chromatogram of *lyso*-ganglioside and *lyso*-sulfatide

4.4. Sample Preparation

The sample preparation described in *3.5.*, worked very well for *lyso*-glycosphingolipids (psychosine, glucosylsphingosine, *lyso*-Gb3) and *lyso*-sphingomyelin, but not for *lyso*-gangliosides and *lyso*-sulfatide. The solid phase extraction (SPE) as described in 3.5. was not optimal for *lyso*-gangliosides and *lyso*-sulfatide. So the method was modified a bit for the *lyso*-gangliosides and *lyso*-sulfatide which also eluted from the cartridges in the second washing step. Therefore also the second washing step was collected. Furthermore it was tested to eluate the *lyso*-gangliosides and *lyso*-sulfatide with 1200 µl of 2% FA in methanol instead of 0.2 % FA in methanol. The washing step was collected together with the eluate and evaporated to dryness under nitrogen at 37°C. The dried samples were resolved in 100 µl of mobile phase B and then transferred to vials for LC-MS/MS analysis. With the described preparation method a qualitative analysis was possible, but no quantitative determination of *lyso*-ganglioside and *lyso*-sulfatide, because of the lack of an adequate internal standard. Three different internal standards were tested the D_3GM2, D_3GM1 and the N-Hexadecanoyl-sulfatide, but the internal standards had a different elution behaviour then the *lyso*-gangliosides and *lyso*-sulfatide. The *lyso*-gangliosides and *lyso*-sulfatide, with the described solid phase extraction had a recovery of around 50 %. Whereas the tested internal standard had the following recoveries, D_3GM2 had 41 %, D_3GM1 had 20 % and the N-Hexadecanoyl-sulfatide had 21 %. With the elution step of 1200 µl of 2% FA in methanol the *lyso*-gangliosides, *lyso*-sulfatide and the internal standard D_3GM_2 had a recovery of around 30 %.

4.5. Quantification

To determine the concentrations of psychosine, glucosylsphingosine, *lyso*-Gb3 and *lyso*-sphingomyelin in unknown plasma samples, calibration curves for each accumulating substance were set up as described in *3.6. Calibration, quantification and method validation*. Furthermore the limit of detection (LOD) and the limit of quantification (LOQ) for each calibration was determined. Also normal values and the reproducibility of normal values of psychosine, glucosylsphingosine, *lyso*-Gb3 and *lyso*-sphingomyelin in plasma were specified. In addition some plasma samples of Fabry and Krabbe patients were analysed.

4.5.1. Psychosine and Glucosylsphingosine

To determine the concentrations of psychosine and glucosylsphingosine, in unknown samples, two separate calibration curves were set up as described in *3.6.1. Psychosine and Glucosylsphingosine*.

In figure 25 the calibration curves of psychosine and glucosylsphingosine are shown. Both calibration curves are linear from 100 to 3.125 ng/ml. Both calibration curves are weighted $1/x^2$, because the lower standard concentration are more important for the determination of normal values in plasma samples.

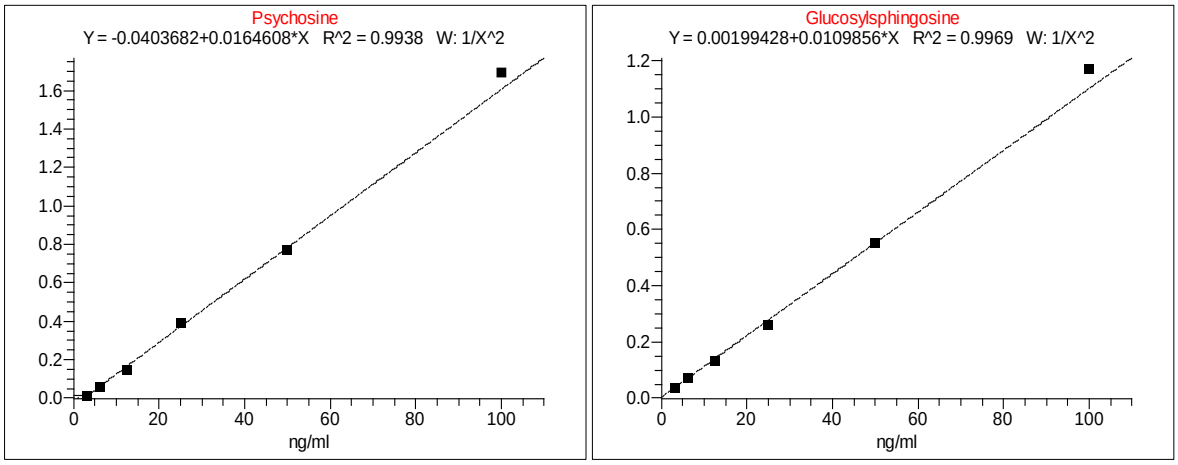

Figure 25: Calibration curve of psychosine (left) and glucosylsphingosine (right)

LOD and LOQ of the psychosine and glucosylsphingosine calibration

The LOD and LOQ of the psychosine and glucosylsphingosine calibration were determined as described in *3.6.1. Psychosine and Glucosylsphingosine*. The parameters needed for the

calculation of LOD and LOQ are given in table 14. The results of LOD and LOQ for psychosine and glucosylsphingosine are shown in table 15.

Table 14: Mean and standard deviation of psychosine and glucosylsphingosine for the calculation of LOD and LOQ

	Psychosine	Glucosylsphingosine
Mean	0.0617631	0.0699747
Standard deviation	0.0030316	0.0021910
Slope of calibration curve	0.0164608	0.0109856

Table 15: Calculated LOD and LOQ of psychosine and glucosylsphingosine

	Psychosine	Glucosylsphingosine
Limit of detection (LOD)	0.553 ng/ml	0.598 ng/ml
Limit of quantification (LOQ)	1.842 ng/ml	1.994 ng/ml

It can be seen that the limits of quantification are lower than the lowest calibration standard. The lowest standard in the calibration curve has a concentration of 3.125 ng/ml. The following standard would have a concentration of 1.562 ng/ml, which is lower than the LOQ. Therefore the standard is excluded in the calibration curve.

The normal values of healthy controls are, for psychosine and glucosylsphingosine, below 3 ng/ml and it is not possible anymore with the established method to distinguish whether it is psychosine or glucosylsphingosine.

4.5.2. *lyso*-Glycosphingolipids and *lyso*-Sphingomyelin

To determine the concentrations of psychosine, glucosylsphingosine, *lyso*-globotriaosylsphingosine and *lyso*-sphingomyelin, in unknown samples, three calibration curves were set up as described in *3.6.2. lyso-Glycosphingolipids and lyso-Sphingomyelin*.

lyso-Globotriaosylsphingosine

In figure 26 the calibration curve of *lyso*-globotriaosylsphingosine (*lyso*-Gb3 or *lyso*-CTH) is shown. The calibration curve is linear from 50 to 0.098 ng/ml and weighted $1/x^2$, because the lower standard concentration are more important for the determination of normal values in plasma samples.

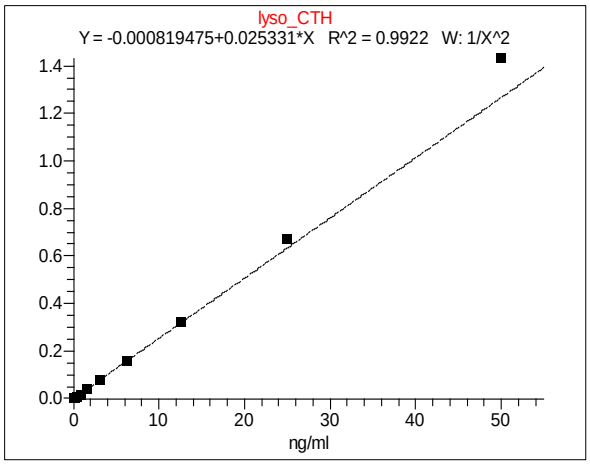

Figure 26: Calibration curve of lyso-Gb3

Lyso-HexosylCer

In figure 27 the calibration curve of *lyso*-HexosylCer is shown. The calibration curve is linear from 100 to 0.195 ng/ml and weighted $1/x^2$, because the lower standard concentration are more important for the determination of normal values in plasma samples.

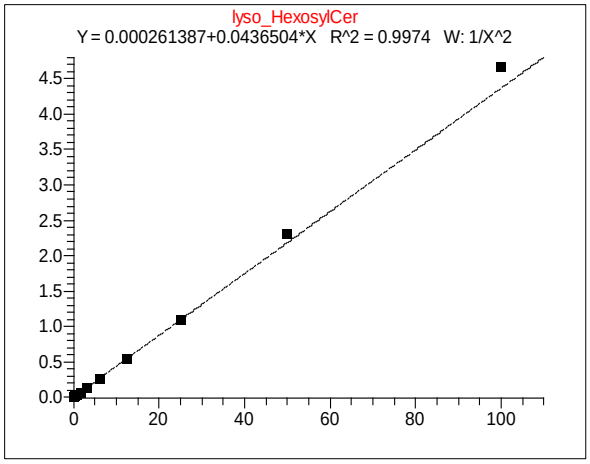

Figure 27: Calibration curve of *lyso*-HexosylCer

lyso-Sphingomyelin

In figure 28 the calibration curve of *lyso*-sphingomyelin is shown. The calibration curve is not linear from 69.552 to 2.174 ng/ml, but it was not possible to get a linear calibration curve in any concentration range.

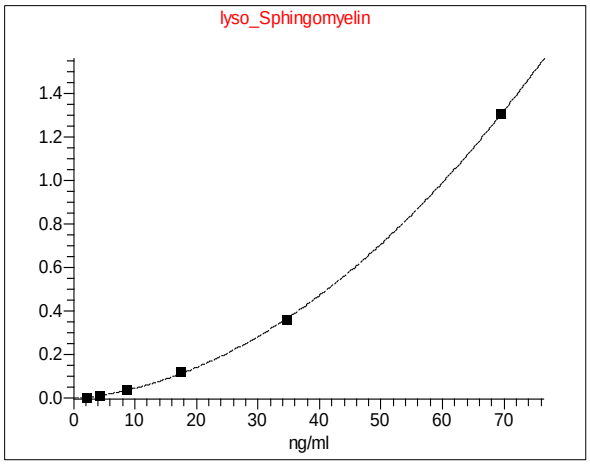

Figure 28: Calibration curve of *lyso*-sphingomyelin

LOD and LOQ of lyso-Glycosphingolipids and lyso-Sphingomyelin calibration

The LOD and LOQ of the *lyso*-Gb3, *lyso*-HexosylCer and *lyso*-Sphingomyelin calibrations were determined as described in *3.6.2. lyso-Glycosphingolipids and lyso-Sphingomyelin*. The parameters needed for the calculation are given in table 16. The results of LOD and LOQ for *lyso*-Gb3, *lyso*-HexosylCer and *lyso*-Sphingomyelin are shown in table 17.

Table 16: Mean and Standard deviation of *lyso*-Gb3, *lyso*-sphingomyelin and *lyso*-HexosylCer

	***lyso*-Gb3**	***lyso*-Sphingomyelin**	***lyso*-HexosylCer**
Mean	0.0783251	0.0101680	0.27510611
Standard deviation	0.0012296	0.0010892	0.00238719
Slope of calibration curve	0.0253310	0.0025938	0.0436504

Table 17: LOD and LOQ of *lyso*-Gb3, *lyso*-sphingomyelin and *lyso*-HexosylCer

	***lyso*-Gb3**	***lyso*-Sphingomyelin**	***lyso*-HexosylCer**
Limit of detection (LOD)	0.146 ng/ml	1.260 ng/ml	0.164 ng/ml
Limit of quantification (LOQ)	0.485 ng/ml	4.199 ng/ml	0.547 ng/ml

In table 18 the reproducibility, in form of the coefficient of variation, of sample preparation and analysis of *lyso*-Gb3, *lyso*-HexosylCer and *lyso*-Sphingomyelin, as described in *3.6.2. lyso-Glycosphingolipids and lyso-Sphingomyelin*, is shown.

Table 18: Reproducibility of the *lyso*-Gb3, *lyso*-sphingomyelin and *lyso*-HexosylCer sample preparation and analysis of healthy controls (n=6)

	***lyso*-Gb3**	***lyso*-Sphingomyelin**	***lyso*-HexosylCer**
Mean	0.380 ng/ml	23.556 ng/ml	0.614 ng/ml
Standard deviation	0.045 ng/ml	0.555 ng/ml	0.017 ng/ml
Coefficient of variation	11.84 %	2.36 %	2.77%

In table 19 the normal values of *lyso*-Gb3, *lyso*-HexosylCer and *lyso*-Sphingomyelin in healthy controls, as described in *3.6.2. lyso-Glycosphingolipids and lyso-Sphingomyelin.*, are shown.

Table 19: Normal values of *lyso*-Gb3, *lyso*-sphingomyelin and *lyso*-HexosylCer, of healthy controls (n=10)

	***lyso*-Gb3**	***lyso*-Sphingomyelin**	***lyso*-HexosylCer**
Mean	0.447 ng/ml	24.165 ng/ml	0.656 ng/ml
Standard deviation	0.225 ng/ml	5.279 ng/ml	0.179 ng/ml

It can be seen that the normal values of *lyso*-Gb3 are quite low and the mean concentrations of the healthy controls is between the LOD and LOQ. The normal mean value of *lyso*-HexosylCer is a bit above the LOQ. In comparison to *lyso*-Gb3 and *lyso*-HexosylCer the normal values of *lyso*-Sphingomyelin are quite elevated.

Fabry and Krabbe patients

The results of the analysed Fabry and Krabbe plasma samples are shown in table 20. The samples 1 to 6, shown in the table below are all samples with an elevated *lyso*-Gb3 concentration. The samples, with the sample number 1 to 3 are controls from a ring trail, were *lyso*-Gb3 is elevated. The samples with the numbers 4 to 6 are plasma samples from Fabry patients treated with ERT. The last one with the sample number 7 is a plasma sample from a Krabbe patient. The samples with an elevated *lyso*-Gb3 concentration were analysed with the new established method (described in *3.4.2.*) and with an already existing and validated *lyso*-Gb3 method from LKH Graz (existing *lyso*-Gb3 method). The results of the *lyso*-Gb3 concentrations with the new established method are shown in the second column and the results of the "existing *lyso*-Gb3 method" are shown in the third column in red.

Table 20: Results of analysed Fabry and Krabbe plasma samples with new established method and the existing *lyso*-Gb3 method (red column)

Sample number	***lyso*-Gb3 [ng/ml]**	***lyso*-Gb3 (existing *lyso*-Gb3 method) [ng/ml]**	***lyso*-Sphingomyelin [ng/ml]**	***lyso*-HexosylCer [ng/ml]**
1	84.018	81	-	-
2	85.212	93.50	-	-
3	42.995	45.07	-	-
4	13.170	12.11	13.151	0.642
5	9.266	8.41	23.573	0.704
6	20.600	30.70	38.163	0.510
7	0.147	-	28.556	10.172

5. Discussion

The analysis and quantification of the synthesised *lyso*-GM2 and *lyso*-GM3 was done to identify the purification fractions with the highest yield and to determine their concentrations. Furthermore, the different species of *lyso*-GM2 and *lyso*-GM3 contained in the standards and their corresponding adducts which are formed in the ESI process, were determined. This was necessary to use them for further analysis and method development. The *lyso*-GM2 standard was synthesised from purchased GM2 (natural, human Tay-Sachs). The analysis showed that there are two different species, *lyso*-GM2 (d18:1) and *lyso*-GM2 (d20:1), contained in the standard. The contained species refer to the natural source of GM2, which is human Tay-Sachs. As mentioned in *2.2. Structure and function of sphingolipids and glycosphingolipids*, the two sphingoid bases (d18:1) and (d20:1) are the most common ones in human. Whereas the analysis of the *lyso*-GM3 standard shows that there is only one species, *lyso*-GM3 (d18:1), contained. This could be due to the natural source of GM3, which is bovine buttermilk. The yields of the synthesis were 1.50 % for *lyso*-GM2 and 2.56 % for *lyso*-GM3. The yield of the *lyso*-GM3 synthesis is higher, although less enzyme was used for the synthesis in comparison to the synthesis of *lyso*-GM2. Ito et al. (*39*) postulated a higher specificity of sphingolipid ceramide N-deacylase for GM2, which is also in contrast to the result of higher yield for *lyso*-GM3. Therefore it is possible that the higher yield for *lyso*-GM3 occurs due to the different purification methods. Probably the purification method 1 is more efficient than method 2, because the *lyso*-GM2 was eluted in every collected fraction, whereas in method 1 the *lyso*-GM3 elution was limited to fraction 5, of the eluate.

Furthermore, also the purchased standards and internal standards were analysed to identify the different species and their corresponding adducts, contained in the standards. The psychosine standard was semisynthetic from bovine. The analysis of psychosine showed that there is only one species, the psychosine (d18:1), contained in the standard. There is a corresponding adduct of psychosine formed which was identified as the Na-adduct. The glucosylsphingosine standard was also semisynthetic, but form bovine buttermilk. The analysis of glucosylsphingosine showed that there are to different species contained in the standard, the glucosylsphingosine (d16:1) and (d18:1) and a Na-adduct of both species is formed. The *lyso*-Gb3 standard was semisynthetic from porcine (red blood cells). The analysis of *lyso*-Gb3 showed that there is only one species, the *lyso*-Gb3 (d18:1) and the corresponding Na-adduct, contained in the standard.

The *lyso*-sphingomyelin standard was semisynthetic from bovine buttermilk. The analysis of *lyso*-sphingomyelin showed that there are two species, the *lyso*-sphingomyelin (d16:1) and (d18:1) and their corresponding Na-adducts, contained in the standard. The *lyso*-GM1 standard was semisynthetic from bovine. The analysis showed that there are two different species, the *lyso*-GM1 (d18:1) and (d20:1), contained in the standard. There are also adducts formed which were identified as sodium formate clusters. The GM2 standard as mentioned was natural from human Tay-Sachs. The analysis of GM2 showed that there are two different species, the GM2 (d18:1) and (d20:1), contained in the standard. The standard D_3GM2 was semisynthetic from human Tay-Sachs. The analysis of D_3GM2 showed that there is only one species, the D_3GM2 (d18:1/18:0), contained in the standard. The GM3 standard was natural from bovine buttermilk. The analysis showed that there are a few different species contained in the standard. The different species in the GM3 standard are all sphingosines (d18:1) with N-linked fatty acids in different chain lengths. The following GM3 species (d18:1/16:0), (d18:1/21:0), (d18:1/22:0), (d18:1/23:0), (d18:1/24:0), are contained in the standard. The standards according to their source contain different species, but also similarities in the species pattern of standards from the same source can be seen. For example the standards semisynthetic from bovine buttermilk, glucosylsphingosine and *lyso*-sphingomyelin, contain the same sphingoid base pattern. Furthermore, the GM2 standard natural from human Tay-Sachs contains, as mentioned above, the two most common sphingoid bases in human. The GM3 standard natural from bovine contains a very broad pattern of species in comparison to the standards which are semisynthetic.

The aim was to develop a single LC-MS/MS method analysing the different accumulating *lyso*-sphingolipids which are characteristic for each lysosomal storage diseases (LSD). It was not possible to cover all lysosomal storage disease, GM1-gangliosidosis, Tay-Sachs disease, Sandhoff disease, Fabry disease, Gaucher disease, Krabbe disease, metachromatic leukodystrophy and Niemann-Pick disease within a single screening method. Therefore three different methods were established for analysing the different accumulating *lyso*-sphingolipids in patients suffering from a lysosomal storage disease.

The accumulation products psychosine and glucosylsphingosine only differ in a steric OH-group on the carbohydrate headgroups. It is not possible to separate them according to their *m/z*, the differentiation of these two substance can only be achieved with chromatographic separation.

With the developed method in *3.4.1. Psychosine and Glucosylsphingosine*, no baseline separation was achieved, (shown in *4.3.1.*, figure 20), but it is possible to identify whether in a sample psychosine or glucosylsphingosine is contained, because in plasma samples of LSD patients never both substances are elevated. It is either a patient with Krabbe disease and elevated level of psychosine or a patient with Gaucher disease and elevated level of glucosylsphingosine. With the internal standard glucosylsphingosine (plant), which only is differentiated from glucosylsphingosine in an additional double bond in the sphingosine chain, the quantification is possible. The monoisotopic mass of glucosylsphingosine (plant) is 2 *m/z* lower than the mass of psychosine and glucosylsphingosine. Therefore it is also possible to distinguish psychosine and glucosylsphingosine, because glucosylsphingosine (plant) and glucosylsphingosine have the same retention behaviour, whereas the psychosine, according to the internal standard is shifted backwards (shown in *4.3.1.*, figure 21). The sample preparation described in *3.5.*, worked well for psychosine, glucosylsphingosine and the internal standard. Also the calibration with two separate calibration curves, as described in *3.6.1.*, showed good results. The limit of detection (LOD) was 0.55 ng/ml for psychosine and 0.60 ng/ml for glucosylsphingosine. The determined limit of quantification (LOQ) was 1.8 ng/ml for psychosine and 2.0 ng/ml for glucosylsphingosine. But between the LOD and LOQ it was not possible anymore to distinguish between psychosine and glucosylsphingosine. The differentiation is just achieved above the LOQ. The determined normal values for healthy controls were below the level of the last standard of 3.125 ng/ml and it was not possible to distinguish between psychosine and glucosylsphingosine. The postulated normal values for healthy controls from literature are <3 ng/ml for psychosine (42). The value for healthy controls is equal to our result.

The *lyso*-glycosphingolipids, *lyso*-HexosylCer, *lyso*-Gb3 and *lyso*-sphingomyelin are similar in retention and ionisation behaviour, and were analysed within a single method, described in *3.4.2.*. The chromatographic separation is shown in *4.3.2.*, figure 22. All substances are adequate separated and were identified over their *m/z*. Only the separation of *lyso*-HexosylCer (psychosine and glucosylsphingosine) is not achieved within the method. Psychosine and glucosylsphingosine, as mentioned before have to be separated with the method described in *3.4.1.*. An occurred abnormality in the chromatographic separation, shown in *4.3.2.*, figure 22, is that all peaks, accept *lyso*-Gb3, occur as double peaks. Probably these double peaks refer to

two different cis/trans isomers. Furthermore, the substances which occur as double peaks are all semisynthetic from bovine, whereas the source of *lyso*-Gb3 is porcine (red blood cells). Maybe there is a connection between the sources of the standards and their containing isomer forms. The sample preparation described in *3.5.*, worked well for *lyso*-HexosylCer, *lyso*-Gb3, *lyso*-sphingomyelin and the internal standard (*lyso*-LC). Also for the quantification good results were achieved. The calibration curves of *lyso*-HexosylCer, *lyso*-Gb3 were linear, whereas no linear calibration curve was achieved for *lyso*-sphingomyelin. It is not clear why it was not possible to achieve a linear calibration curve for *lyso*-sphingomyelin, probably it is due to the ionisation behaviour of the different species contained in the standard. This calibration definitely has to be repeated. The results for the limit of detection were 0.15 ng/ml for *lyso*-Gb3, 0.16 ng/ml for *lyso*-HexosylCer and 1.3 ng/ml for *lyso*-sphingomyelin. The determined limits of quantification were 0.49 ng/ml for *lyso*-Gb3, 0.55 ng/ml for *lyso*-HexosylCer and 4.2 ng/ml for *lyso*-sphingomyelin. To determine the reproducibility of the sample preparation and analysis, a single plasma sample was analysed for 6 times. The results of the reproducibility, in form of the dispersion around the mean value, are 12 % for *lyso*-Gb3, 2.8 % for *lyso*-HexosylCer and 2.4 % for *lyso*-sphingomyelin. The dispersion around the mean value for the low concentrations in healthy patients is quite good. The results for healthy controls (n=10) were 0.45±0.23 ng/ml for *lyso*-Gb3 which is lower than the LOQ, 0.66±0.18 ng/ml for *lyso*-HexosylCer and 24±5.3 ng/ml for *lyso*-sphingomyelin. The postulate normal values from literature are 2.09 nmol/l (0.96 ng/ml) for *lyso*-HexosylCer (*9*) and 0.7 nmol/l (0.55 ng/ml) for *lyso*-Gb3 (*41*). These literature values are equal to our values found in healthy controls. Whereas the literature normal values for *lyso*-sphingomyelin are 10.26 nmol/l (*9*) and 10.3 nmol/l (4,8ng/ml) (*21*). In comparison to our findings the normal values for *lyso*-sphingomyelin are five times higher than the postulated ones from literature. This could be due to the quadratic calibration which has to be repeated.

Also a few plasma samples from patients with Fabry or Krabbe were analysed and compared to the already existing and validated *lyso*-Gb3 method from LKH Graz., shown in *4.5.2.*, table 20. The samples 1-3 with elevated *lyso*-Gb3 were from a ring trail and the results with the new established method were compared to the results of the "existing *lyso*-Gb3 method". The result of the sample concentrations below 50 ng/ml fitted well to each other. Whereas the sample concentrations in the new established method above 50 ng/ml vary from these analysed with the "existing *lyso*-Gb3 method". This is probably due to that the last standard concentration is 50

ng/ml in the new established method. Therefor the concentrations of the samples 1-2 are out of the calibration range. The samples 4-6 were samples from patients suffering from Fabry disease, but treated with enzyme replacement therapy (ERT). The determined plasma concentrations of *lyso*-Gb3 with the new established method fit to the results of the "existing *lyso*-Gb3 method". In literature also *lyso*-Gb3 plasma concentrations of ERT treated patients were found. The postulated literature mean value is 40.2 nmol/l (31.6 ng/ml) for *lyso*-Gb3 (*41*), which in the quite in the same scale in comparison to our findings, 14 ng/ml for *lyso*-Gb3. But it is expected that the values of treated patients differ, because it depends on how long they are receiving the ERT and how well the therapy is adopted. Only a single sample of a Krabbe patient was analysed. The result was 10 ng/ml for *lyso*-HexosylCer. In literature the following concentration levels, in blood pots, were found, 23-73 ng/ml for patients with infantile Krabbe disease and 1.7-5.7 ng/ml for patients with low GALC activity (*42*). Our result is between these two levels, but also in the same range.

The chromatographic separation of gangliosides (*lyso*-GM1, GM2, GM3), *lyso*-sulfatide and internal standard D_3GM2 was achieved with the method described in *3.4.3. Gangliosides and lyso-Sulfatide.* The chromatogram for *lyso*-GM1, GM2, *lyso*-sulfatide and internal standard is shown in *4.3.3*., figure 23. The peaks are well shaped and separated. In the chromatogram only a single peak for GM2 (d18:1/18:0) was found, but no peak for GM2 (d20:1/18:1), which also was identified in *4.2. Structure analysis of standards*. This is due to the timed inclusion list, because the time windows are very close and probably the RT of GM2 (d20:1/18:0) is out of the time window range. The chromatograms for the GM3 species are shown in *4.3.3.*, figure 25 and 26. In the GM3 standard there are seven different species contained and for some species double or triple peaks occur. Probably this is a chromatographic occurrence or there are different isomers contained in the standard. The separation of gangliosides and lyso-sulfatide was only a test, if it is possible to separate the different species within a single run. Actually the substances of interest were the *lyso*-forms.

The chromatographic separation of *lyso*-gangliosides, *lyso*-sulfatide and internal standard D_3GM2 was achieved with the method described in *3.4.4. lyso-Gangliosides and lyso-Sulfatide.* The chromatogram for *lyso*-GM1, *lyso*-GM2, *lyso*-GM3, *lyso*-Sulfatide and internal standard is shown in *4.3.4.*, figure 26 and the peaks are well shaped and separated. For the quantification

different sample preparations and internal standards were tried, but the sample preparation described in 3.5., and also modified versions of the sample preparation did not lead to the desired result. The *lyso*-gangliosides and *lyso*-sulfatide had different elution behaviours in comparison to the tested internal standards. The most suitable tried internal standard was D_3GM2. The D_3GM3 standard was not tested, probably it is an alternative to the tested ones or the sample preparation has to be adapted to the substances. Because the sample preparation, especially the SPE, described in *3.5.*, is adjusted for basic compounds, but the gangliosides also have a N-acetylneuraminic acid attached. Therefore no quantitative analysis for *lyso*-gangliosides and *lyso*-sulfatide was achieved.

In conclusion for all substances of interest a LC-MS/MS method and a sample preparation suitable for a part of the substances of interest, was developed. Instead of a single LC-MS/MS method, three different methods to cover all lysosomal storage disorders were developed, because the accumulating substances are quite diverse in retention and ionisation behaviour. With the developed LC-MS/MS methods, furthermore it is possible to distinguish between patients with Gaucher disease and Krabbe disease and it is also possible to determine the concentration of the accumulating *lyso*-glycosphingolipids of these diseases. Further on it is possible to quantify the accumulating products of Fabry and Niemann-Pick, with one of the developed LC-MS/MS methods. The lyso-gangliosides and lyso-sulfatide were detected, but it was not possible to quantify them, because of the lack of an adequate internal standard. In summary there is still a lot of work to finally establish the screening method in the laboratory.

6. References

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7. Abbreviations

ACN	acetonitrile
AGC target	automatic gain control
BMP	bis(monoacylglycero)phosphate
BSA	bovine serum albumin
Cer	ceramide
CERT	ceramide transfer protein
CNS	central nervous system
CRE	charged residue model
D_3GM2	N-omega-CD_3-Octadecanoyl monosialoganglioside GM2
DNA	deoxyribonucleic acid
EGF	epithelial growth factor
ER	endoplasmic reticulum
ERT	enzyme replacement therapy
ESI	electrospray ionisation
FA	formic acid
FFT	fast Fourier transformation
GalCer	galactosylceramide
GalNAc	N-acetylgalactosamine
Gb3	globotriaosylceramide
GC	gas chromatography
GlcCer	glucosylceramide
GLY	glucosylsphingosine
GLY plant	glucosylsphingosine plant
GM1	Monosialoganglioside GM1
GM2	Monosialoganglioside GM2
GM2-AP	GM2- activator protein
GM3	Monosialoganglioside GM3
GT	gene therapy
H_2O	water

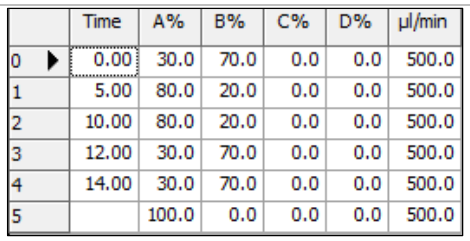
HILIC	hydrophilic interaction liquid chromatography
HPLC	high performance liquid chromatography
HR-MS	high resolution-mass spectrometry
HSCT	hematopoietic stem cell transplantation
IEM	ion evaporation model
IST	internal standard
LacCer	lactosylceramide
LC-MS	liquid chromatography-mass spectrometry
LSD	lysosomal storage disease
lyso-Gb3	*lyso*-Globotriaosylsphingosine
lyso-GM1	*lyso*-Monosialoganglioside GM1
lyso-GM2	*lyso*-Monosialoganglioside GM2
lyso-GM3	*lyso*-Monosialoganglioside GM3
lyso-HexosylCer	psychosine and glucosylsphingosine
lyso-LC	*lyso*-lactosylceramide
M6-P	mannose 6-phosphate
MeOH	methanol
MLD	metachromatic leukodystrophy
MPS	mucopolysaccharidosis
MSX count	spectral multiplexing
NDP	Niemann-Pick disease
Neu5A	N-acetylneuraminic acid
NPC1/NPC2	Niemann-Pick disease type C1/C2 (intracellular cholesterol transporter)
NP-LC	normal phase-liquid chromatography
PCT	pharmacological chaperon therapy
PDGF	platelet derived growth factor
PGC	porous graphitic carbon
PNS	peripheral nervous system
PSY	psychosine
RNA	ribonucleic acid
RP-LC	reversed phase liquid chromatography

rpm	revolutions per minute
RT	room temperature/ retention time
SAP	sphingolipid activator protein
SPC	*lyso*-Sphingomyelin
SPE	solid phase extraction
SRT	substrate reduction therapy
TNG	trans-Golgi network
TOF	time of flight

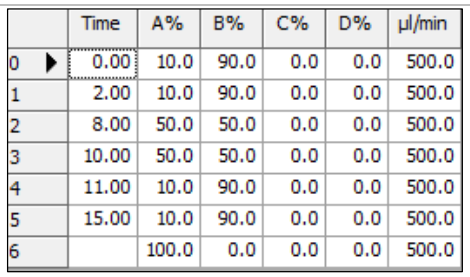
8. Figures and Tables

9. Appendix

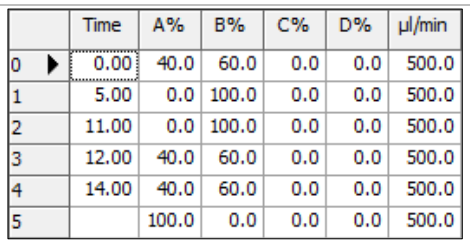
9.1. Psychosine and Glucosylsphingosine

Method Name	**Psychosine_M1_18.03.2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 350-600 *m/z*

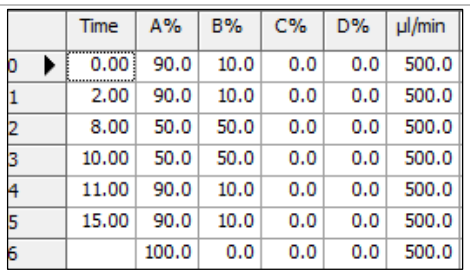
	Time	A%	B%	C%	D%	µl/min
0	0.00	30.0	70.0	0.0	0.0	500.0
1	5.00	80.0	20.0	0.0	0.0	500.0
2	10.00	80.0	20.0	0.0	0.0	500.0
3	12.00	30.0	70.0	0.0	0.0	500.0
4	14.00	30.0	70.0	0.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M2_18.03.2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 350-600 *m/z*

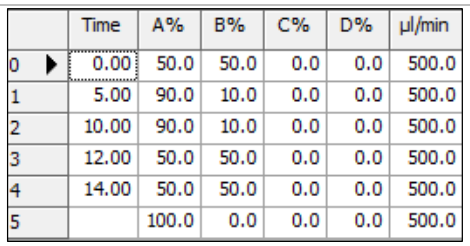
	Time	A%	B%	C%	D%	µl/min
0	0.00	10.0	90.0	0.0	0.0	500.0
1	2.00	10.0	90.0	0.0	0.0	500.0
2	8.00	50.0	50.0	0.0	0.0	500.0
3	10.00	50.0	50.0	0.0	0.0	500.0
4	11.00	10.0	90.0	0.0	0.0	500.0
5	15.00	10.0	90.0	0.0	0.0	500.0
6		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_testC18_23_03_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD C 18 1.9 µm (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 250-600 *m/z*

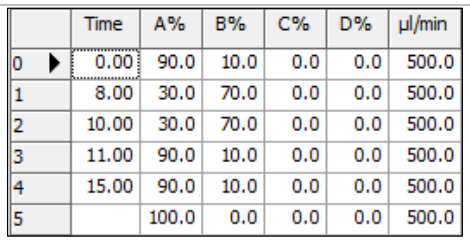
	Time	A%	B%	C%	D%	µl/min
0	0.00	40.0	60.0	0.0	0.0	500.0
1	5.00	0.0	100.0	0.0	0.0	500.0
2	11.00	0.0	100.0	0.0	0.0	500.0
3	12.00	40.0	60.0	0.0	0.0	500.0
4	14.00	40.0	60.0	0.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M3_29_03_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 250-600 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	90.0	10.0	0.0	0.0	500.0
1	2.00	90.0	10.0	0.0	0.0	500.0
2	8.00	50.0	50.0	0.0	0.0	500.0
3	10.00	50.0	50.0	0.0	0.0	500.0
4	11.00	90.0	10.0	0.0	0.0	500.0
5	15.00	90.0	10.0	0.0	0.0	500.0
6		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M4_29_03_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 250-600 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	50.0	50.0	0.0	0.0	500.0
1	5.00	90.0	10.0	0.0	0.0	500.0
2	10.00	90.0	10.0	0.0	0.0	500.0
3	12.00	50.0	50.0	0.0	0.0	500.0
4	14.00	50.0	50.0	0.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M5_30_03_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 250-600 *m/z*

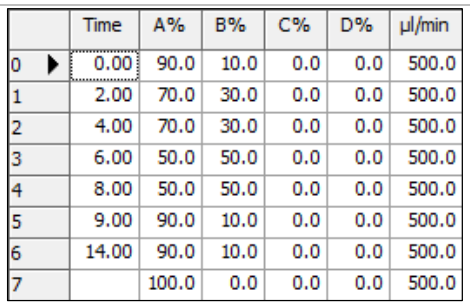
	Time	A%	B%	C%	D%	µl/min
0	0.00	90.0	10.0	0.0	0.0	500.0
1	8.00	30.0	70.0	0.0	0.0	500.0
2	10.00	30.0	70.0	0.0	0.0	500.0
3	11.00	90.0	10.0	0.0	0.0	500.0
4	15.00	90.0	10.0	0.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M6_30_03_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 250-600 *m/z*

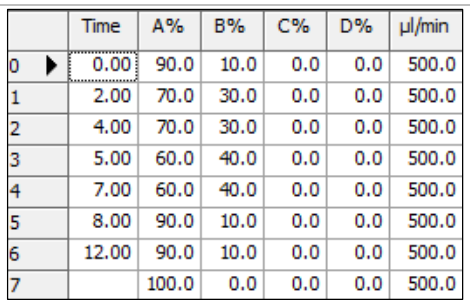
	Time	A%	B%	C%	D%	µl/min
0	0.00	90.0	10.0	0.0	0.0	500.0
1	6.00	30.0	70.0	0.0	0.0	500.0
2	8.00	30.0	70.0	0.0	0.0	500.0
3	9.00	90.0	10.0	0.0	0.0	500.0
4	14.00	90.0	10.0	0.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M7_30_03_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 250-600 *m/z*

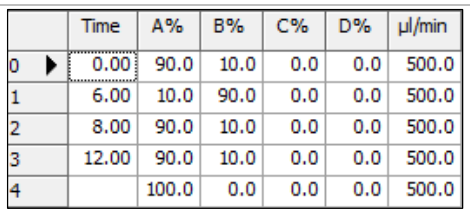
	Time	A%	B%	C%	D%	µl/min
0	0.00	90.0	10.0	0.0	0.0	500.0
1	3.00	60.0	40.0	0.0	0.0	500.0
2	5.00	60.0	40.0	0.0	0.0	500.0
3	7.00	50.0	50.0	0.0	0.0	500.0
4	9.00	50.0	50.0	0.0	0.0	500.0
5	10.00	90.0	10.0	0.0	0.0	500.0
6	15.00	90.0	10.0	0.0	0.0	500.0
7		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M8_31_03_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 250-600 *m/z*

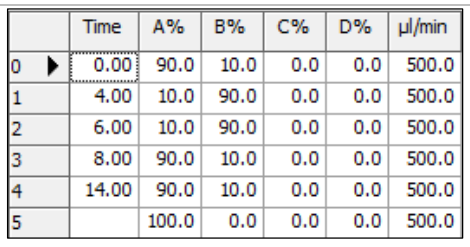
	Time	A%	B%	C%	D%	µl/min
0	0.00	90.0	10.0	0.0	0.0	500.0
1	2.00	70.0	30.0	0.0	0.0	500.0
2	4.00	70.0	30.0	0.0	0.0	500.0
3	6.00	50.0	50.0	0.0	0.0	500.0
4	8.00	50.0	50.0	0.0	0.0	500.0
5	9.00	90.0	10.0	0.0	0.0	500.0
6	14.00	90.0	10.0	0.0	0.0	500.0
7		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M9_31_03_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 250-600 *m/z*

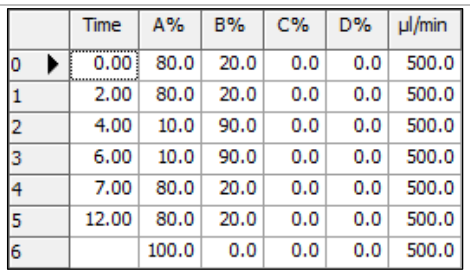
	Time	A%	B%	C%	D%	µl/min
0	0.00	90.0	10.0	0.0	0.0	500.0
1	2.00	70.0	30.0	0.0	0.0	500.0
2	4.00	70.0	30.0	0.0	0.0	500.0
3	5.00	60.0	40.0	0.0	0.0	500.0
4	7.00	60.0	40.0	0.0	0.0	500.0
5	8.00	90.0	10.0	0.0	0.0	500.0
6	12.00	90.0	10.0	0.0	0.0	500.0
7		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M10_31_03_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Flow rate	500 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 250-600 *m/z*

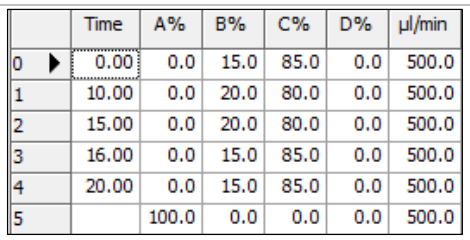
	Time	A%	B%	C%	D%	µl/min
0	0.00	90.0	10.0	0.0	0.0	500.0
1	6.00	10.0	90.0	0.0	0.0	500.0
2	8.00	90.0	10.0	0.0	0.0	500.0
3	12.00	90.0	10.0	0.0	0.0	500.0
4		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M11_31_03_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 350-600 *m/z*

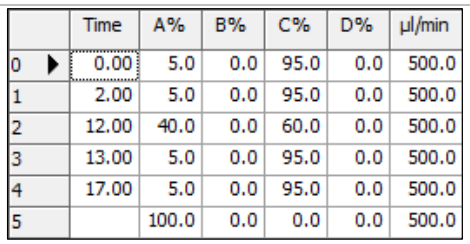
	Time	A%	B%	C%	D%	µl/min
0	0.00	90.0	10.0	0.0	0.0	500.0
1	4.00	10.0	90.0	0.0	0.0	500.0
2	6.00	10.0	90.0	0.0	0.0	500.0
3	8.00	90.0	10.0	0.0	0.0	500.0
4	14.00	90.0	10.0	0.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M12_31_03_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 400-600 *m/z*

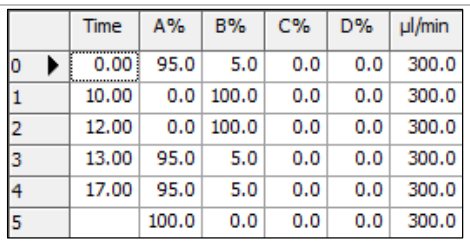
	Time	A%	B%	C%	D%	µl/min
0	0.00	80.0	20.0	0.0	0.0	500.0
1	2.00	80.0	20.0	0.0	0.0	500.0
2	4.00	10.0	90.0	0.0	0.0	500.0
3	6.00	10.0	90.0	0.0	0.0	500.0
4	7.00	80.0	20.0	0.0	0.0	500.0
5	12.00	80.0	20.0	0.0	0.0	500.0
6		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M13_11_04_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 350-600 *m/z*

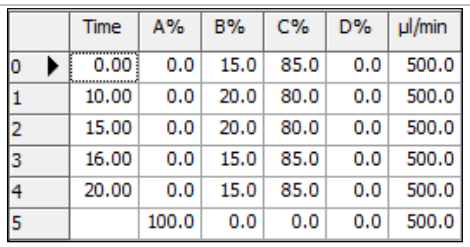
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	500.0
1	10.00	0.0	20.0	80.0	0.0	500.0
2	15.00	0.0	20.0	80.0	0.0	500.0
3	16.00	0.0	15.0	85.0	0.0	500.0
4	20.00	0.0	15.0	85.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M14_11_04_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 350-600 *m/z*

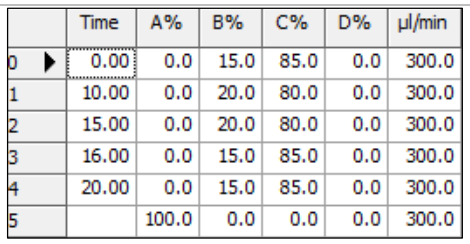
	Time	A%	B%	C%	D%	µl/min
0	0.00	5.0	0.0	95.0	0.0	500.0
1	2.00	5.0	0.0	95.0	0.0	500.0
2	12.00	40.0	0.0	60.0	0.0	500.0
3	13.00	5.0	0.0	95.0	0.0	500.0
4	17.00	5.0	0.0	95.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M15_PGC_15_04_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Thermo Scientific Hypercarb 3 µm (100 x 3 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 400-500 *m/z*

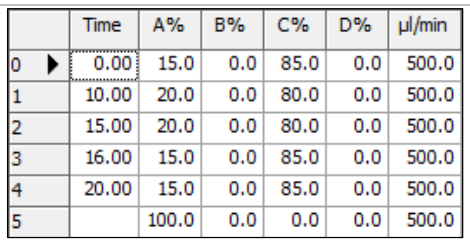
	Time	A%	B%	C%	D%	µl/min
0	0.00	95.0	5.0	0.0	0.0	300.0
1	10.00	0.0	100.0	0.0	0.0	300.0
2	12.00	0.0	100.0	0.0	0.0	300.0
3	13.00	95.0	5.0	0.0	0.0	300.0
4	17.00	95.0	5.0	0.0	0.0	300.0
5		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M16_19_04_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 mS; scan range: 450-500 *m/z*

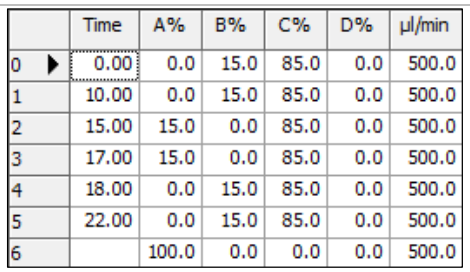
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	500.0
1	10.00	0.0	20.0	80.0	0.0	500.0
2	15.00	0.0	20.0	80.0	0.0	500.0
3	16.00	0.0	15.0	85.0	0.0	500.0
4	20.00	0.0	15.0	85.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M16_300_19_04_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 450-500 *m/z*

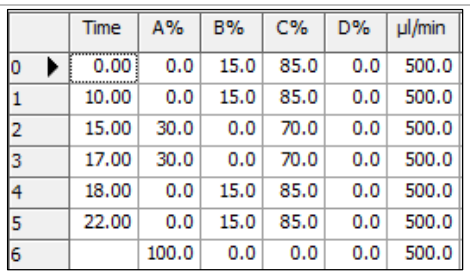
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	10.00	0.0	20.0	80.0	0.0	300.0
2	15.00	0.0	20.0	80.0	0.0	300.0
3	16.00	0.0	15.0	85.0	0.0	300.0
4	20.00	0.0	15.0	85.0	0.0	300.0
5		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M17_20_04_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 450-500 *m/z*

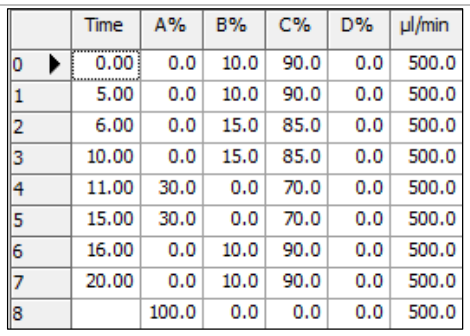
	Time	A%	B%	C%	D%	µl/min
0	0.00	15.0	0.0	85.0	0.0	500.0
1	10.00	20.0	0.0	80.0	0.0	500.0
2	15.00	20.0	0.0	80.0	0.0	500.0
3	16.00	15.0	0.0	85.0	0.0	500.0
4	20.00	15.0	0.0	85.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M18_22_04_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 450-500 *m/z*

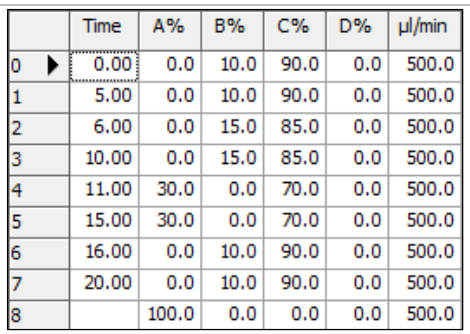
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	500.0
1	10.00	0.0	15.0	85.0	0.0	500.0
2	15.00	15.0	0.0	85.0	0.0	500.0
3	17.00	15.0	0.0	85.0	0.0	500.0
4	18.00	0.0	15.0	85.0	0.0	500.0
5	22.00	0.0	15.0	85.0	0.0	500.0
6		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M19_02_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 450-490 *m/z*

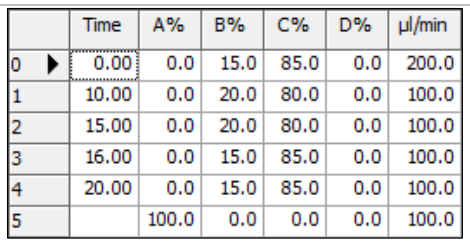
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	500.0
1	10.00	0.0	15.0	85.0	0.0	500.0
2	15.00	30.0	0.0	70.0	0.0	500.0
3	17.00	30.0	0.0	70.0	0.0	500.0
4	18.00	0.0	15.0	85.0	0.0	500.0
5	22.00	0.0	15.0	85.0	0.0	500.0
6		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M20_02_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 450-500 *m/z*

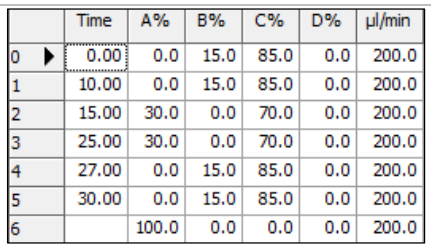
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	10.0	90.0	0.0	500.0
1	5.00	0.0	10.0	90.0	0.0	500.0
2	6.00	0.0	15.0	85.0	0.0	500.0
3	10.00	0.0	15.0	85.0	0.0	500.0
4	11.00	30.0	0.0	70.0	0.0	500.0
5	15.00	30.0	0.0	70.0	0.0	500.0
6	16.00	0.0	10.0	90.0	0.0	500.0
7	20.00	0.0	10.0	90.0	0.0	500.0
8		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M21_02_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 450-490 *m/z*

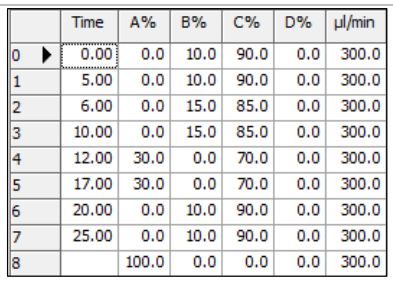
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	10.0	90.0	0.0	500.0
1	5.00	0.0	10.0	90.0	0.0	500.0
2	6.00	0.0	15.0	85.0	0.0	500.0
3	10.00	0.0	15.0	85.0	0.0	500.0
4	11.00	30.0	0.0	70.0	0.0	500.0
5	15.00	30.0	0.0	70.0	0.0	500.0
6	16.00	0.0	10.0	90.0	0.0	500.0
7	20.00	0.0	10.0	90.0	0.0	500.0
8		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M22_06_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 455-490 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	200.0
1	10.00	0.0	20.0	80.0	0.0	100.0
2	15.00	0.0	20.0	80.0	0.0	100.0
3	16.00	0.0	15.0	85.0	0.0	100.0
4	20.00	0.0	15.0	85.0	0.0	100.0
5		100.0	0.0	0.0	0.0	100.0

Method Name	**Psychosine_M23_06_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 450-490 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	200.0
1	10.00	0.0	15.0	85.0	0.0	200.0
2	15.00	30.0	0.0	70.0	0.0	200.0
3	25.00	30.0	0.0	70.0	0.0	200.0
4	27.00	0.0	15.0	85.0	0.0	200.0
5	30.00	0.0	15.0	85.0	0.0	200.0
6		100.0	0.0	0.0	0.0	200.0

Method Name	**Psychosine_M24_06_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 450-490 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	10.0	90.0	0.0	300.0
1	5.00	0.0	10.0	90.0	0.0	300.0
2	6.00	0.0	15.0	85.0	0.0	300.0
3	10.00	0.0	15.0	85.0	0.0	300.0
4	12.00	30.0	0.0	70.0	0.0	300.0
5	17.00	30.0	0.0	70.0	0.0	300.0
6	20.00	0.0	10.0	90.0	0.0	300.0
7	25.00	0.0	10.0	90.0	0.0	300.0
8		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M25_06_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 450-490 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	10.00	0.0	15.0	85.0	0.0	300.0
2	15.00	15.0	0.0	85.0	0.0	300.0
3	25.00	15.0	0.0	85.0	0.0	300.0
4	27.00	0.0	15.0	85.0	0.0	300.0
5	30.00	0.0	15.0	85.0	0.0	300.0
6		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M26_09_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 450-490 *m/z*

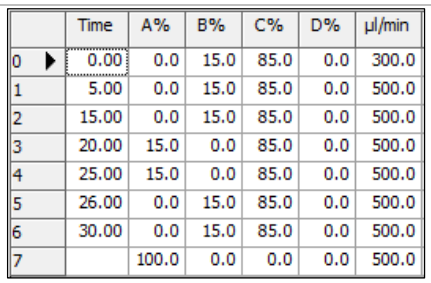
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	5.00	0.0	15.0	85.0	0.0	300.0
2	15.00	0.0	30.0	70.0	0.0	300.0
3	25.00	0.0	30.0	70.0	0.0	300.0
4	26.00	0.0	15.0	85.0	0.0	300.0
5	30.00	0.0	15.0	85.0	0.0	300.0
6		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M27_09_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 455-490 *m/z*

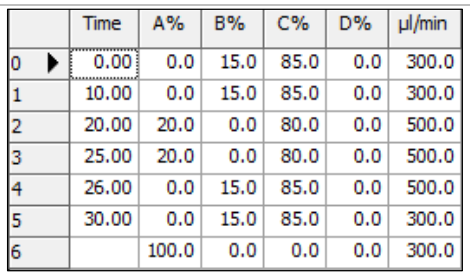
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	500.0
1	10.00	0.0	15.0	85.0	0.0	500.0
2	20.00	0.0	20.0	80.0	0.0	500.0
3	25.00	0.0	20.0	80.0	0.0	500.0
4	26.00	0.0	15.0	85.0	0.0	500.0
5	30.00	0.0	15.0	85.0	0.0	500.0
6		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M28_10_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 450-510 *m/z*

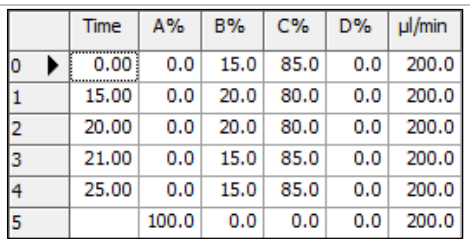
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	10.0	90.0	0.0	500.0
1	5.00	0.0	15.0	85.0	0.0	500.0
2	10.00	0.0	15.0	85.0	0.0	500.0
3	20.00	0.0	30.0	70.0	0.0	500.0
4	25.00	0.0	30.0	70.0	0.0	500.0
5	26.00	0.0	10.0	90.0	0.0	500.0
6	30.00	0.0	10.0	90.0	0.0	500.0
7		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M29_10_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 455-490 *m/z*

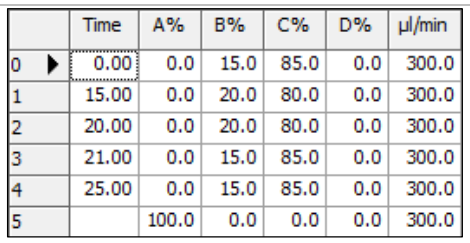
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	5.00	0.0	15.0	85.0	0.0	500.0
2	15.00	0.0	15.0	85.0	0.0	500.0
3	20.00	15.0	0.0	85.0	0.0	500.0
4	25.00	15.0	0.0	85.0	0.0	500.0
5	26.00	0.0	15.0	85.0	0.0	500.0
6	30.00	0.0	15.0	85.0	0.0	500.0
7		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_M30_10_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 455-490 *m/z*

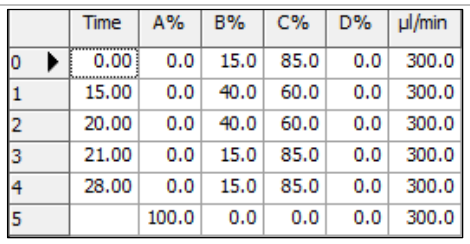
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	10.00	0.0	15.0	85.0	0.0	300.0
2	20.00	20.0	0.0	80.0	0.0	500.0
3	25.00	20.0	0.0	80.0	0.0	500.0
4	26.00	0.0	15.0	85.0	0.0	500.0
5	30.00	0.0	15.0	85.0	0.0	300.0
6		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M22_S4_11_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 455-490 *m/z*

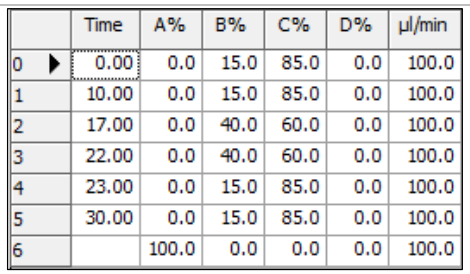
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	200.0
1	15.00	0.0	20.0	80.0	0.0	200.0
2	20.00	0.0	20.0	80.0	0.0	200.0
3	21.00	0.0	15.0	85.0	0.0	200.0
4	25.00	0.0	15.0	85.0	0.0	200.0
5		100.0	0.0	0.0	0.0	200.0

Method Name	**Psychosine_M22_S4300_11_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 455-490 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	15.00	0.0	20.0	80.0	0.0	300.0
2	20.00	0.0	20.0	80.0	0.0	300.0
3	21.00	0.0	15.0	85.0	0.0	300.0
4	25.00	0.0	15.0	85.0	0.0	300.0
5		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M31_11_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 455-490 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	15.00	0.0	40.0	60.0	0.0	300.0
2	20.00	0.0	40.0	60.0	0.0	300.0
3	21.00	0.0	15.0	85.0	0.0	300.0
4	28.00	0.0	15.0	85.0	0.0	300.0
5		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M32_S4100_12_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 455-490 *m/z*

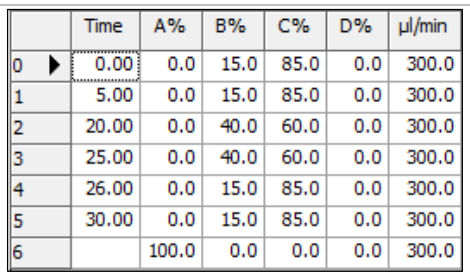
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	100.0
1	10.00	0.0	15.0	85.0	0.0	100.0
2	17.00	0.0	40.0	60.0	0.0	100.0
3	22.00	0.0	40.0	60.0	0.0	100.0
4	23.00	0.0	15.0	85.0	0.0	100.0
5	30.00	0.0	15.0	85.0	0.0	100.0
6		100.0	0.0	0.0	0.0	100.0

Method Name	**Psychosine_M32_S4300_12_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 455-490 *m/z*

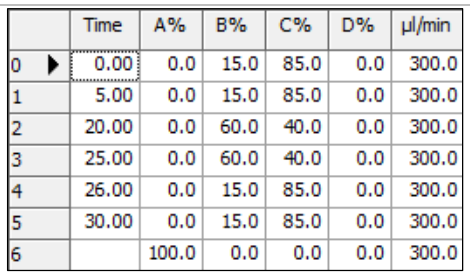
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	10.00	0.0	15.0	85.0	0.0	300.0
2	17.00	0.0	40.0	60.0	0.0	300.0
3	22.00	0.0	40.0	60.0	0.0	300.0
4	23.00	0.0	15.0	85.0	0.0	300.0
5	30.00	0.0	15.0	85.0	0.0	300.0
6		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M33_S4300_12_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 ms; scan range: 455-490 *m/z*

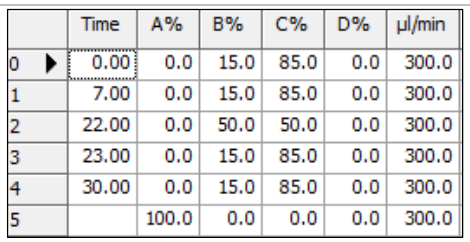
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	7.00	0.0	15.0	85.0	0.0	300.0
2	17.00	0.0	50.0	50.0	0.0	300.0
3	22.00	0.0	50.0	50.0	0.0	300.0
4	23.00	0.0	15.0	85.0	0.0	300.0
5	30.00	0.0	15.0	85.0	0.0	300.0
6		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M34_12_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-490 *m/z*

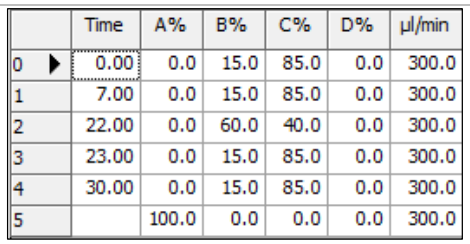
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	5.00	0.0	15.0	85.0	0.0	300.0
2	20.00	0.0	40.0	60.0	0.0	300.0
3	25.00	0.0	40.0	60.0	0.0	300.0
4	26.00	0.0	15.0	85.0	0.0	300.0
5	30.00	0.0	15.0	85.0	0.0	300.0
6		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M35_13_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-490 *m/z*

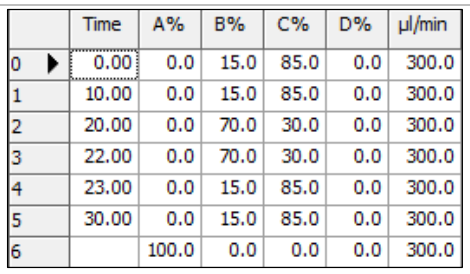
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	5.00	0.0	15.0	85.0	0.0	300.0
2	20.00	0.0	60.0	40.0	0.0	300.0
3	25.00	0.0	60.0	40.0	0.0	300.0
4	26.00	0.0	15.0	85.0	0.0	300.0
5	30.00	0.0	15.0	85.0	0.0	300.0
6		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M36_13_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-490 *m/z*

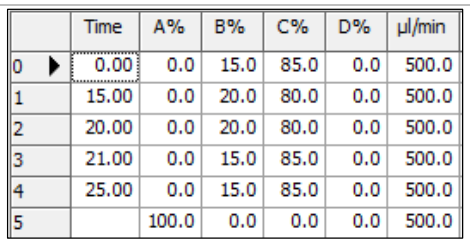
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	7.00	0.0	15.0	85.0	0.0	300.0
2	22.00	0.0	50.0	50.0	0.0	300.0
3	23.00	0.0	15.0	85.0	0.0	300.0
4	30.00	0.0	15.0	85.0	0.0	300.0
5		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M37_13_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-490 *m/z*

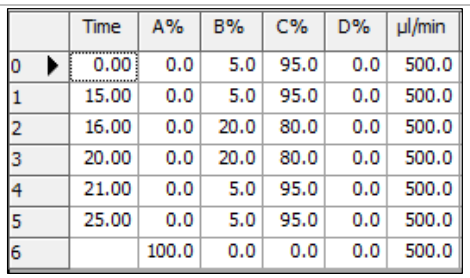
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	7.00	0.0	15.0	85.0	0.0	300.0
2	22.00	0.0	60.0	40.0	0.0	300.0
3	23.00	0.0	15.0	85.0	0.0	300.0
4	30.00	0.0	15.0	85.0	0.0	300.0
5		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_M38_13_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm) Phenomenex Kinetex 2.6 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-490 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	300.0
1	10.00	0.0	15.0	85.0	0.0	300.0
2	20.00	0.0	70.0	30.0	0.0	300.0
3	22.00	0.0	70.0	30.0	0.0	300.0
4	23.00	0.0	15.0	85.0	0.0	300.0
5	30.00	0.0	15.0	85.0	0.0	300.0
6		100.0	0.0	0.0	0.0	300.0

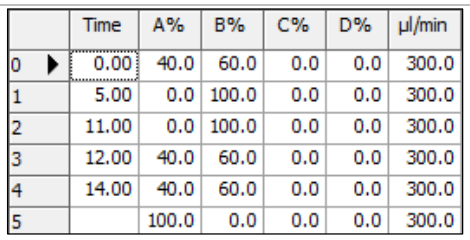
Method Name	**Psychosine_M22_3S500_13_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	40°C
Column	2x Phenomenex Kinetex 1.7 µm HILIC 100A (50 x 2.1 mm) Phenomenex Kinetex 1.7 µm HILIC 100A (100 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-490 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	500.0
1	15.00	0.0	20.0	80.0	0.0	500.0
2	20.00	0.0	20.0	80.0	0.0	500.0
3	21.00	0.0	15.0	85.0	0.0	500.0
4	25.00	0.0	15.0	85.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

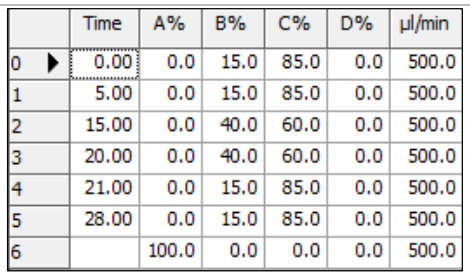
Method Name	**Psychosine_CN_M2_23_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD CN 3 µm (150 x 4 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-490 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	5.0	95.0	0.0	500.0
1	15.00	0.0	5.0	95.0	0.0	500.0
2	16.00	0.0	20.0	80.0	0.0	500.0
3	20.00	0.0	20.0	80.0	0.0	500.0
4	21.00	0.0	5.0	95.0	0.0	500.0
5	25.00	0.0	5.0	95.0	0.0	500.0
6		100.0	0.0	0.0	0.0	500.0

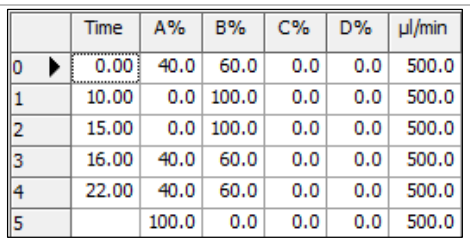
9.2. *lyso*-Glucosylsphingosine and *lyso*-Sphingomyelin

Method Name	**Psychosine_C18_17_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD C 18 1.9 µm (50 x 2.1 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-850 *m/z*

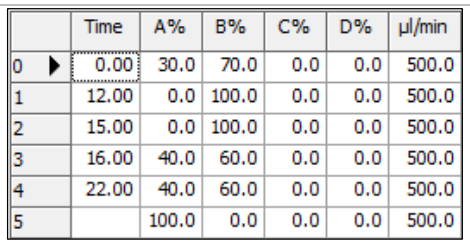
	Time	A%	B%	C%	D%	µl/min
0	0.00	40.0	60.0	0.0	0.0	300.0
1	5.00	0.0	100.0	0.0	0.0	300.0
2	11.00	0.0	100.0	0.0	0.0	300.0
3	12.00	40.0	60.0	0.0	0.0	300.0
4	14.00	40.0	60.0	0.0	0.0	300.0
5		100.0	0.0	0.0	0.0	300.0

Method Name	**Psychosine_CN_M1_23_05_2016**
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD CN 3 µm (150 x 4 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-850 *m/z*

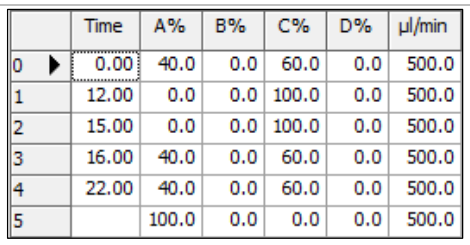
	Time	A%	B%	C%	D%	µl/min
0	0.00	0.0	15.0	85.0	0.0	500.0
1	5.00	0.0	15.0	85.0	0.0	500.0
2	15.00	0.0	40.0	60.0	0.0	500.0
3	20.00	0.0	40.0	60.0	0.0	500.0
4	21.00	0.0	15.0	85.0	0.0	500.0
5	28.00	0.0	15.0	85.0	0.0	500.0
6		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_aQ_M1_25_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-850 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	40.0	60.0	0.0	0.0	500.0
1	10.00	0.0	100.0	0.0	0.0	500.0
2	15.00	0.0	100.0	0.0	0.0	500.0
3	16.00	40.0	60.0	0.0	0.0	500.0
4	22.00	40.0	60.0	0.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_aQ_M2_25_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-850 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	30.0	70.0	0.0	0.0	500.0
1	12.00	0.0	100.0	0.0	0.0	500.0
2	15.00	0.0	100.0	0.0	0.0	500.0
3	16.00	40.0	60.0	0.0	0.0	500.0
4	22.00	40.0	60.0	0.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_aQ_M3_30_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-850 *m/z*

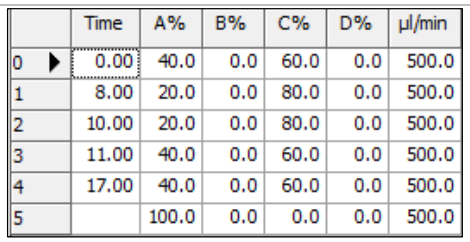
	Time	A%	B%	C%	D%	µl/min
0	0.00	40.0	0.0	60.0	0.0	500.0
1	12.00	0.0	0.0	100.0	0.0	500.0
2	15.00	0.0	0.0	100.0	0.0	500.0
3	16.00	40.0	0.0	60.0	0.0	500.0
4	22.00	40.0	0.0	60.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Psychosine_aQ_M4_31_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-850 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	30.0	0.0	70.0	0.0	500.0
1	10.00	15.0	0.0	85.0	0.0	500.0
2	14.00	15.0	0.0	85.0	0.0	500.0
3	15.00	0.0	0.0	100.0	0.0	500.0
4	19.00	0.0	0.0	100.0	0.0	500.0
5	20.00	30.0	0.0	70.0	0.0	500.0
6	24.00	30.0	0.0	70.0	0.0	500.0
7		100.0	0.0	0.0	0.0	500.0

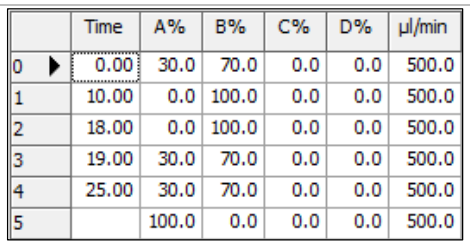
Method Name	**Psychosine_aQ_M3_1_01_06_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-850 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	40.0	0.0	60.0	0.0	500.0
1	12.00	0.0	0.0	100.0	0.0	500.0
2	14.00	0.0	0.0	100.0	0.0	500.0
3	15.00	40.0	0.0	60.0	0.0	500.0
4	20.00	40.0	0.0	60.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

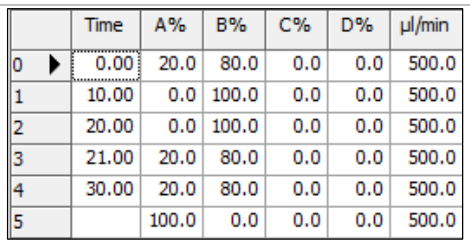
Method Name	**Psychosine_aQ_M5_01_06_05_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase C	0.1% FA/0.01% acetic acid/ACN
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	GB3_lyso_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 455-850 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	40.0	0.0	60.0	0.0	500.0
1	8.00	20.0	0.0	80.0	0.0	500.0
2	10.00	20.0	0.0	80.0	0.0	500.0
3	11.00	40.0	0.0	60.0	0.0	500.0
4	17.00	40.0	0.0	60.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

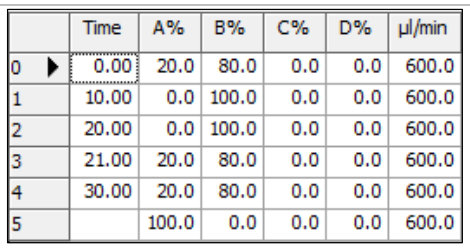
9.3. Gangliosides and *lyso*-Sulfatide

Method Name	**Sphingosine_Neg_aQ_M1_04_08_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	Ganglioside_neg_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 520-1700 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	30.0	70.0	0.0	0.0	500.0
1	10.00	0.0	100.0	0.0	0.0	500.0
2	18.00	0.0	100.0	0.0	0.0	500.0
3	19.00	30.0	70.0	0.0	0.0	500.0
4	25.00	30.0	70.0	0.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Sphingosine_Neg_aQ_M2_18_08_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	Ganglioside_neg_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 900-1300 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	20.0	80.0	0.0	0.0	500.0
1	10.00	0.0	100.0	0.0	0.0	500.0
2	20.00	0.0	100.0	0.0	0.0	500.0
3	21.00	20.0	80.0	0.0	0.0	500.0
4	30.00	20.0	80.0	0.0	0.0	500.0
5		100.0	0.0	0.0	0.0	500.0

Method Name	**Sphingosine_Neg_aQ_M3_18_08_2016**
Mobile phase A	1.2% FA/0.38% ammonium acetate/H_2O
Mobile phase B	1.2% FA/0.38% ammonium acetate/MeOH
Oven temperature	25°C
Column	Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)
Injection volume	10 µl
Gradient	
Ion source	ESI (electrospray ionization)
Ionization settings	Ganglioside_neg_tune (see 3.3.)
MS/MS	Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 520-1650 *m/z*

	Time	A%	B%	C%	D%	µl/min
0	0.00	20.0	80.0	0.0	0.0	600.0
1	10.00	0.0	100.0	0.0	0.0	600.0
2	20.00	0.0	100.0	0.0	0.0	600.0
3	21.00	20.0	80.0	0.0	0.0	600.0
4	30.00	20.0	80.0	0.0	0.0	600.0
5		100.0	0.0	0.0	0.0	600.0

<table>
<tr><td>Method Name</td><td>Sphingosine_Neg_aQ_M4_19_08_2016</td></tr>
<tr><td>Mobile phase A</td><td>1.2% FA/0.38% ammonium acetate/H_2O</td></tr>
<tr><td>Mobile phase C</td><td>0.1% FA/0.01% acetic acid/ACN</td></tr>
<tr><td>Oven temperature</td><td>25°C</td></tr>
<tr><td>Column</td><td>Thermo Scientific Hypersil GOLD aQ 3 µm (150 x 4 mm)</td></tr>
<tr><td>Injection volume</td><td>10 µl</td></tr>
<tr><td>Gradient</td><td>
<table>
<tr><th></th><th>Time</th><th>A%</th><th>B%</th><th>C%</th><th>D%</th><th>µl/min</th></tr>
<tr><td>0</td><td>0.00</td><td>40.0</td><td>0.0</td><td>60.0</td><td>0.0</td><td>500.0</td></tr>
<tr><td>1</td><td>12.00</td><td>0.0</td><td>0.0</td><td>100.0</td><td>0.0</td><td>500.0</td></tr>
<tr><td>2</td><td>18.00</td><td>0.0</td><td>0.0</td><td>100.0</td><td>0.0</td><td>500.0</td></tr>
<tr><td>3</td><td>19.00</td><td>40.0</td><td>0.0</td><td>60.0</td><td>0.0</td><td>500.0</td></tr>
<tr><td>4</td><td>26.00</td><td>40.0</td><td>0.0</td><td>60.0</td><td>0.0</td><td>500.0</td></tr>
<tr><td>5</td><td></td><td>100.0</td><td>0.0</td><td>0.0</td><td>0.0</td><td>500.0</td></tr>
</table>
</td></tr>
<tr><td>Ion source</td><td>ESI (electrospray ionization)</td></tr>
<tr><td>Ionization settings</td><td>Ganglioside_neg_tune (see 3.3.)</td></tr>
<tr><td>MS/MS</td><td>Full scan; positive; resolution: 70 000; AGC target: $3e^6$; maximum IT: 200 m; scan range: 530-1650 m/z</td></tr>
</table>