UNIVERSITY OF PARDUBICE

FACULTY OF CHEMICAL TECHNOLOGY

Department of Analytical Chemistry

Mgr. Karel Hořejší

Comprehensive analysis of glycosphingolipids in biological samples using HPLC/MS

Theses of the Doctoral Dissertation

Pardubice 2024

Study program: **Analytical Chemistry**

Study field: **Analytical Chemistry**

Author: **Mgr. Karel Hořejší**

Supervisor: **prof. Ing. Michal Holčapek, Ph.D.**

Year of the defence: 2024

Reference

HOŘEJŠÍ, Karel. *Comprehensive characterization of glycosphingolipids in biological samples using HPLC/MS*. Pardubice, 2024. 381 pages. Dissertation thesis (PhD.). University of Pardubice, Faculty of Chemical Technology, Department of Analytical Chemistry. Supervisor: Prof. Ing. Michal Holčapek, Ph.D.

Abstract

Lipids are biomolecules found in all living organisms, where they have several vital functions. Lipids are the subject of lipidomics, a subgroup of metabolomics, which represents one of the so-called “omics” disciplines that also includes genomics, proteomics, and glycomics. Dysregulation of lipid metabolism can reflect the onset of various diseases including cancer, therefore, lipidomic analysis can provide valuable information about ongoing pathophysiological processes in humans.

The theoretical part provides an extensive overview of glycosphingolipids, including their biosynthesis, structure complexity, classification, nomenclature, and various biological functions in association with pathophysiological conditions as a hallmark of a variety of diseases, for example, cancer. Special attention is also paid to the sample preparation and key analytical methods for their identifications and quantitation. Qualitative and quantitative approaches are discussed as well, along with their advantages and limitations.

The experimental part deals with the development, optimization, and application of analytical methods for the analysis of especially glycosphingolipids in biological samples, such as human plasma/serum and tissues using chromatographic techniques coupled to mass spectrometry. Special attention is devoted to in-depth structural characterization and qualitative glycosphingolipids profiling using a lipid class separation approach (HILIC). Correspondingly, glycosphingolipid profiling is used to expand the database of lipids that are routinely analyzed, and for the mutual comparison of healthy volunteers and cancer patients. Therefore, the analysis of glycosphingolipids has the potential to facilitate the discovery of novel biomarkers for the early detection of various diseases.

Abstrakt

Lipidy jsou biomolekuly, které se nacházejí ve všech živých systémech, kde mají několik životně důležitých funkcí. Lipidy jsou předmětem zkoumání lipidomiky, podskupiny metabolomiky, která reprezentuje jednu z takzvaných omických disciplín, které také zahrnují genomiku, proteomiku a glykomiku. Dysregulace metabolismu lipidů může odrážet nástup různých onemoncnění včetně rakoviny, lipidomická analýza tak může poskytnout cenné informace o probíhajících patofyziologických procesech u lidí.

Teoretická část disertační práce poskytuje rozsáhlý přehled glykosfingolipů, včetně jejich biosyntézy, strukturní složitosti, klasifikace, názvosloví a různých biologických funkcí ve spojení s patofyziologickými stavy, které jsou charakteristickým znakem řady nemocnění, například rakoviny. Zvláštní pozornost je také věnována přípravě vzorků a klíčovým analytickým metodám pro jejich identifikaci a kvantifikaci. Diskutovány jsou rovněž kvalitativní a kvantitativní přístupy společně s jejich výhodami a omezeními.

Experimentální část práce se zabývá vývojem, optimalizací a aplikací analytických metod pro analýzu zejména glykosfinigolipidů v biologických vzorcích, jako je lidská plasma/serum a tkáně, a to s pomocí chromatografických technik ve spojení s hmotnostní spektrometrií. Zvláštní pozornost je věnována hloubkové strukturní charakterizaci a kvalitativnímu profilování glykosfingolipidů za použití přístupu separace lipidových tříd (HILIC). V souladu s tím je profilování glykosfingolipidů využito k rozšíření databáze lipidů, jež jsou rutinně analyzovány a ke vzájemnému srovnání zdravých dobrovolníků a pacientů s rakovinou. Analýza glykosfingolipidů má tedy potenciál usnadnit objev nových biomarkerů pro včasnou detekcí různých onemocnění.

Keywords
lipidomics, glycosphingolipids, plasma, pancreatic tissue, extraction, purification, structural characterization, tandem mass spectrometry, hydrophilic interaction liquid chromatography

Klíčová slova
lipidomika, glykosfingolipidy, plasma, pankreatická tkáň, extrakce, purifikace, strukturní charakterizace, tandemová hmotnostní spektrometrie, hydrofilní interakční kapalinová chromatografie

Table of Contents

Introduction

1 Glycosphingolipids (GSL)

GSL comprise a group of remarkably heterogeneous biomolecules that are found in essentially all eukaryotes, as well as some prokaryotes and viruses. GSL are ubiquitous membrane components, which are almost exclusively located on the outer leaflet of cell plasma membranes and in intracellular organelles (**Fig. 1**) [1,2]. GSL are also found in biofluids, where they either circulate freely and/or are bound to protein complexes [3].

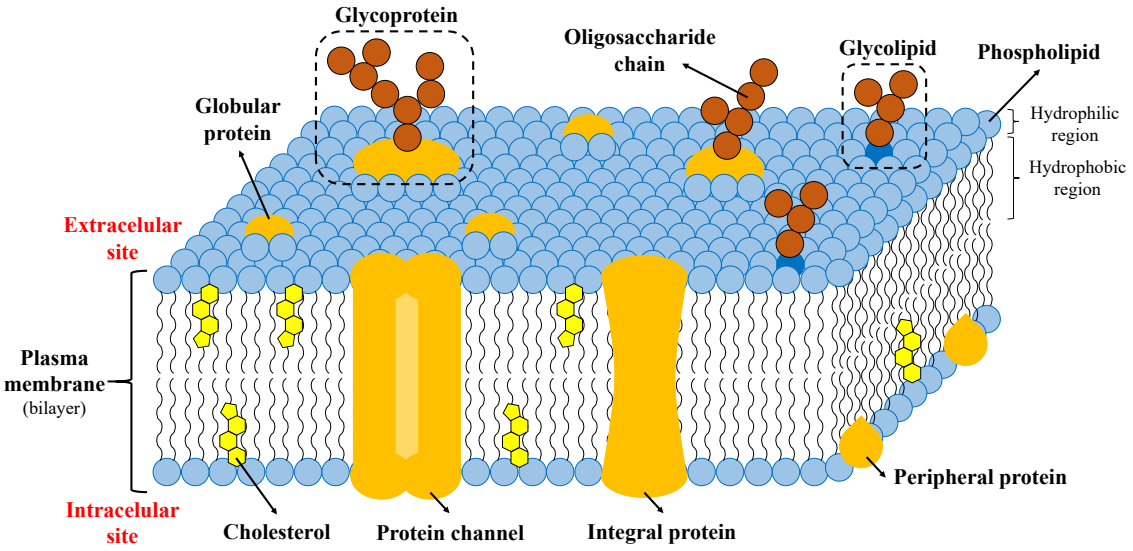

Fig. 1: Cross-section and structure of the cell plasma membrane (modified from [4]).

GSL are amphiphilic molecules composed of two distinct parts. The hydrophobic region consist of ceramide backbone anchored into the plasma membrane, while the hydrophilic region composed of glycan moiety glycosidically linked to a ceramide faces the extracellular environment. The example of GSL structure is illustrated in **Fig. 2** [5]. GSL are classified based on their charges into neutral, acidic, and basic. Neutral GSL (N-GSL) include cerebrosides (1 sugar) and globosides (≥ 2 sugars), while acidic GSL (A-GSL) are subdivided into sialic acid-containing GSL called gangliosides and sulfated GSL called sulfatides. In contrast, basic GSL are rare [2].

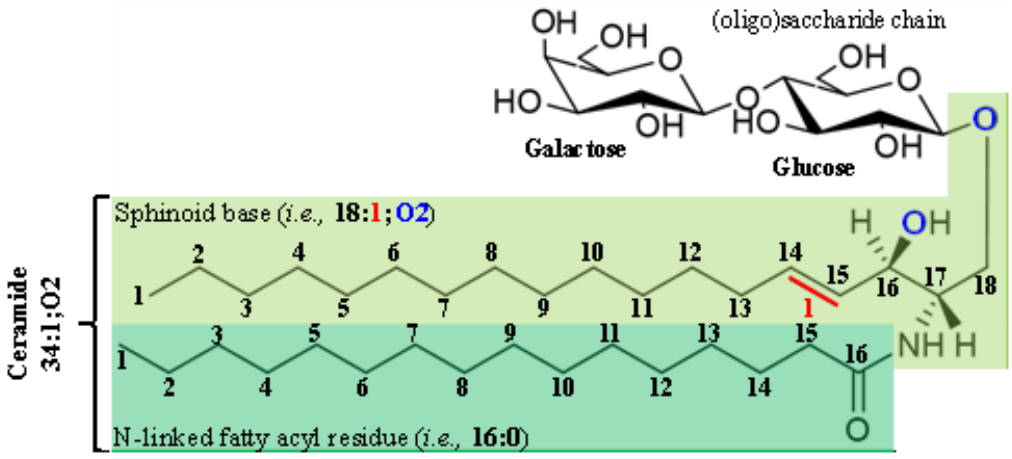

Fig. 2: Example of shorthand annotation of lactosylceramide (LacCer 18:1;O2/16:0).

Besides, the majority of GSL molecules are also classified based on their core structure called “series” (**Table 1**). The major series in vertebrates are ganglio, globo, and neolacto series, whereas in invertebrates the mollu and arthro series with mannose dominate [2,6].

Table 1: Classification of GSL according to their oligosaccharide core and their localization.

GSL series		Predominant occurrence	Core structure			
			IV	III	II	I
Ganglio	**Gg**	brain				
Globo	**Gb**	erythrocytes				
Isoglobo	**iGb**					
Lacto	**Lc**	secretory organs				
Neolacto	**nLc**	hematopoietic cells				
Mollu	**Mu**	invertebrates				
Arthro	**At**	invertebrates				

1.1 Biological functions of GSL

GSL are bioactive effectors with many intriguing and versatile roles in cell physiology and pathophysiology. Their functions are largely governed by the glycan head group. In addition to being major constituents of cell plasma membranes, they also play a role in the organization of membrane microdomains, provide a rigid barrier for the separation of cellular organelles, and are essential for the development of multicellular organisms. GSL also serve as energy storage necessary to ensure many biological processes [5,7]. GSL play a vital role as key transmembrane receptors via protein-protein, carbohydrate-carbohydrate, or protein-carbohydrate interactions. Specifically, GSL may serve as binding ligands or adhesion receptors for various proteins (*e.g.,* antibodies, lectins) [8], cellular molecules (*e.g.,* hormones), microbes (*e.g.,* bacteria and viruses) [9,10], and microbial products (*e.g.,* bacterial toxins) [11,12]. GSL also act as modulators of signal transduction and messengers in cell signaling [13–18].

1.2 Association of GSL with disease

GSL have been shown to be implicated in the pathogenesis of various diseases, such as glycosphingolipidoses, autoimmune diseases, infectious diseases, diabetes, and cardiovascular diseases [19]. It is also well-known that GSL impact cancer cell energy metabolism. A large number of studies have repeatedly reported altered glycosylation patterns as a common feature of carcinogenesis. The dysregulation of glycosyltransferases, namely fucosyltransferases (FUTs) [20] and sialyltransferases (SATs), [21] that are involved in the modification and termination of GSL, have been reported. Thus, aberrant glycosylation with associated enzymes and the shift from type 1 (Lc-series) to type 2 (nLc-series) GSL are now widely accepted as one of the hallmarks of tumor initiation and progression [22]. Fucosylation contributes to the expression of ABH and Lewis antigens in cancer cells, and these antigens may represent potential biomarkers in cancer diagnosis. ABH antigens are typically absent from glyco-lipids/proteins of tumor tissues despite being found in the adjacent normal tissues. This results in the accumulation of H, Le^a, Le^x, their sialyl derivates (*i.e.,* S-Le^a or S-Le^x), and also Le^b and Le^y, which is in line with the increased fucosylation. Consequently, knowledge of the distribution of these antigens in normal tissues is of importance for the evaluation of tumor-associated alterations [23,24].

2 Analysis of GSL

2.1 Extraction

The most predominant extraction method used in lipidomics is liquid-liquid extraction (LLE, **Fig. 3**). The extraction from biological samples is typically accomplished by the use of two-phase LLE based on the partitioning of hydrophobic lipids to an organic layer, while hydrophilic and/or ionic lipids and unwanted molecules (*e.g.,* salts, proteins, nucleic acids, polar metabolites, and cellular debris) are partitioned to an interphase and aqueous layer. A variety of organic solvent mixtures is used for LLE extraction [25]. Namely, the chloroform/methanol-based extraction systems introduced more than a half of century ago by Folch [26] and Bligh-Dyer [27], generally regarded as "the gold standard", are routinely applied. However, these methods do not provide effective recovery of the amphiphilic and highly polar GSL as they generally require more aqueous portion [28,29]. Over the years, several modifications and alternative strategies to these original protocols have been developed. One of them is the method described by Matysh *et al.* [30], which utilizes methyl tert-butyl ether (MTBE) and which was specifically developed for shotgun lipidomics. Furthermore, single-phase butanol-methanol (BUME) extraction system firstly described by Löfgren *et al.* [31,32] and further modified by Alshehry *et al.* [33] has been reported to provide a similar yield of lipids compared to traditional Folch and Bligh-Dyer methods. Interestingly, Vale *et al.* [34] used a three-phase solvent system with two organic layers and one aqueous layer at the bottom for successful extraction of GLs, GPs, and SLs. Unfortunately, lipid species with hydrophilic glycan chains (*e.g.,* gangliosides and sulfatides) or amphiphilic nature (*e.g.,* complex neutral GSL) are mostly partitioned into the aqueous phase and/or are lost to the interphase, limiting the scope of many studies. Consequently, comprehensive lipidomic studies aiming to analyze a wide range of lipids with various polarities typically require more specialized extraction protocols [35].

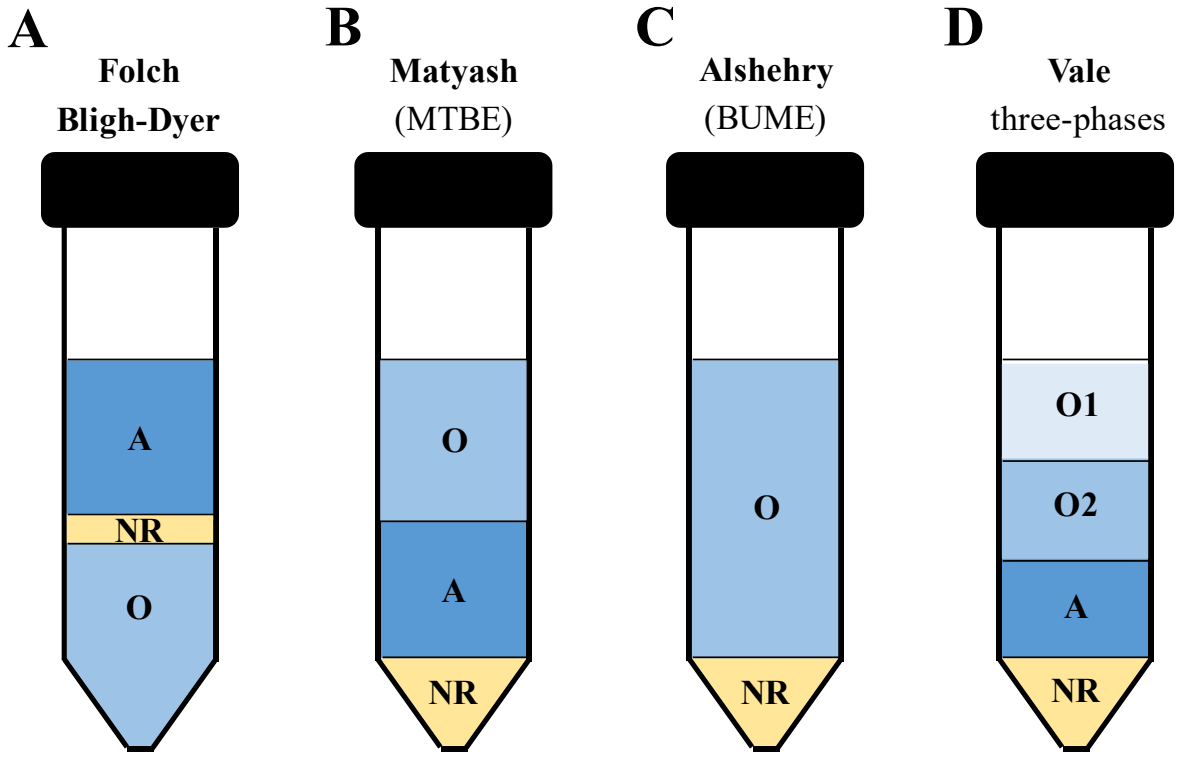

Fig. 3: Schematic illustration of phase separation in common LLE methods (modified from [36] and [34]). A, aqueous phase; O, organic phase; NR, non-extractable residues.

2.2 Solid-phase extraction (SPE)

Solid-phase extraction (SPE) is well-grounded method for the isolation and purification of selected lipids and enrichment of minor lipid classes as well [25]. The most frequently used SPE columns include normal-phase (*e.g.,* silica), reversed-phase (*e.g.,* C8 and C18), and ion-exchange (*e.g.,* aminopropyl) columns. For instance, passing sample through C18 SPE column is the most convenient method removing interfering substances, such as salts and/or other water-soluble non-lipid contaminants [28].

2.3 Mass spectrometry analysis of GSL

There are three major approaches used in lipidomics including direct infusion MS analysis (DI-MS), liquid chromatography or supercritical fluid chromatography coupled to mass spectrometry (LC-MS or SFC-MS), and mass spectrometry imaging (MSI).

2.3.1 Direct infusion mass spectrometry (DI-MS)

DI-MS also termed "shotgun" lipidomics is a technique when lipid extracts are directly introduced into the MS instrument without upfront separation and processed within few minutes in a high-throughput fashion [37]. DI-MS is primarily designed for targeted studies to detect unique intra-source fragments generated by specific lipid (sub)classes. The molecular characterization of lipid species thus relies either on the accurate *m/z* determination of precursor ions (*i.e.,* using a high-resolution mass spectrometry; HRMS) or on the detection of specific product ions or neutral losses in MS^n experiments (*i.e.,* using a low-resolution mass spectrometry; LRMS). Nevertheless, the HRMS instruments are preferred due to their ability to differentiate the isomeric and isobaric compounds [30,38,39]. Additionally, multi-dimensional MS-based shotgun lipidomics (MDMS-SL) allows the separation of many lipid (sub)classes through selective ionization of certain category of lipids in the ion source (*i.e.,* intra-source separation), even if the lipids are minor [37,40,41]. Although ESI and MALDI are by far the most widely used ion sources in DI-MS, few relatively novel soft ionization technologies, namely DESI [42], laser ablation electrospray ionization (LAESI) [43], and matrix-free laser desorption ionization (LDI) [44], have been successfully applied for the direct analysis of lipids in biological samples without complex sample pretreatment.

2.3.2 LC-MS and SFC-MS

LC-MS and SFC-MS, respectively (U)HPLC-MS and UHPSCF are key and well-established conventional analytical methods in lipidomics. They are powerful tools allowing lipid subclass and/or molecular species separation before MS analysis as well as the analysis of very low abundant lipids. However, care must be taken as there are many structural isomers in the sample and, therefore, a single analysis may lead to misinterpretations. The most frequently used ionization technique is ESI, which is best suited for a wide range of lipids that can be analyzed either in positive or negative ion modes. APCI and APPI are valuable alternatives for less polar lipids, such as MG, DG, TG, and CE [38]. ESI-MS is by far the most frequently used analytical technique utilized successfully in large-scale lipidomic studies due to several significant advantages including high sensitivity, easy coupling with chromatography techniques, and structural details based on the use of tandem mass spectrometers with high mass accuracy [45]. Last but not least, interfacing LC with IM has shown a great potential for lipid isomers separation together with increased selectivity and sensitivity [46].

2.3.3 Mass spectrometry imaging (MSI)

MSI has become a popular and powerful method perfectly designed for the analysis of solid samples with the ability to simultaneously display both spatial distribution and molecular level information. The most frequently used ionization technique employed in MSI is MALDI. However, many other less common ionization techniques, *e.g.,* DESI, LAESI, or secondary ion mass spectrometry (SIMS) with the ability to produce sub-micron spatial resolution by using a tiny probe size, have been applied [47,48]. MALDI is also more tolerant to salts and can even ionize heavily glycosylated GSL, but generally have lower ionization efficiency compared to ESI [29]. MALDI also has a few limitations. It can barely resolve isomers without prior separation and generally experiences high background noise and ion suppression effects due to the formation of matrix clusters that preclude the application of this technology in full lipid profiling in complicated biological samples [29,49].

MALDI coupled to time-of-flight analyzers (MALDI-TOF or MALDI-TOF/TOF) is the most widely used MSI technology applied for rapid *in situ* screening of biomolecules in biological samples to map spatial distribution of individual lipid species [50–52]. Moreover, the comprehensive analysis is limited since the MSI is largely based on the qualitative comparison of healthy and diseased samples [52]. Recently, the coupling of MSI with high-resolution MS involving Orbitrap or Fourier transform ion cyclotron resonance (FT-ICR) has provided deeper insight into the lipidomic complexity of biological samples [53]. One such example is the application of MALDI-Orbitrap using MS/MS spectra to facilitate structural elucidation of even highly complex sulfo-GSL with up to five hexose moieties [54].

3 Aims

The goal of this work is to provide a broad overview of GSL and other lipids together with isolation, purification, and concentration strategies required for their comprehensive identification, characterization and in-depth profiling in various biological samples using (U)HPLC-ESI-MS^2.

3.1 Lipid class separation I – characterization of simple GSL

- Determination of N-GSL and A-GSL using untargeted HILIC-ESI/MS^2
- Optimization of lipid preparation and structural elucidation of GSL
- Generation of lipid species profiles in human plasma

3.2 Lipid class separation II – characterization of complex GSL

- Determination of complex GSL using HPLC-ESI/MS^2 on HILIC and PGC columns
- Differentiation of glycan isomers using endoglycoceramidase digestion
- Mutual comparison of GSL profiles of human normal and tumor pancreatic tissues

3.3 Plasma lipid profiles of three types of cancer

- Differentiation of kidney, breast, and prostate cancer from healthy controls based on lipid profiling
- Discovery of potential screening biomarkers for these cancers

3.4 Review: Analysis of GSL in biological samples

- Recent advances, challenges and future directions in the mass spectrometry analysis of GSL in biological samples

4 Material and methods

The complete set of materials used within my Ph.D. study is described in the published paper. Therefore, only the methods are summarized in this part

4.1 Lipid class separation I – characterization of simple GSL

We developed and optimized extraction protocol using monophasic ethanol–water solvent system in combination with solid phase extraction (SPE) designed for isolation, purification, and concentration of GSL from human plasma samples (250 µl). Briefly, 250 µL of human plasma was homogenized and deproteinized in 3 mL of ethanol using ultrasonic bath at 40 °C for 10 min. Then, 600 µL of deionized water was added, the mixture was vortexed for 1 min and centrifuged at 10,000 rpm for 3 min under ambient conditions. The supernatant containing lipids was collected, evaporated in a heated block at 35 °C with a gentle stream of nitrogen to dryness and redissolved in 1 mL of deionized water. Deproteinized and redissolved plasma was purified and concentrated using SPE, where seven C18-based columns (i.e., DSC-18 (A), DSC-18 (B), DSC- 18Lt, SepPak-tC18. ENVI-18, Strata C18-E, Spe-ed C18/18), one C8-based column (i.e., DSC-8), three polymeric columns (i.e., SDB-L, Strata X, Oasis HLB), one normal phase column (i.e., Diol) and two ZrO2-based colums (i.e., Phree, HybridSPE-phospholipid) from several manufacturers were tested using protocols recommended by vendors. The following parameters were optimized: deproteinization solvent (*i.e.,* acetonitrile, MeCN,; methanol, MeOH; ethanol, EtOH; acetone, ACE), the suitability of SPE column (*i.e.,* normal-phase, reversed-phase, polymeric-phase, and ZrO_2-based sorbents; in total 14 SPE columns were tested), and effect of methanol in the loading step (*i.e.,* 0–20% of MeOH). The optimal results were achieved for EtOH as a protein precipitation reagent in combination with Spe-ed C18/18 SPE cartridge and deionized water in the loading step. Optimized method was then applied to the analysis of human plasma. The analysis was performed on high-performance liquid chromatograph Ultimate 3000 (Thermo) coupled with mass spectrometer detector Velos Pro (Thermo) with dual-pressure linear ion trap analyzer. The separation was accomplished on the Ascentis Si column (150 × 2.1 mm, 2.1 µm, Merck) within 25 min run time using a gradient elution: 0 min—10% of mobile phase B, 7 min—11.4% of B, 16 min—20% of B, 18 min—20% of B, where the mobile phase A was acetonitrile, and the mobile phase B was 10mM ammonium acetate in 10% acetonitrile. Both mobile phases were slightly acidified by the addition of 0.5µL of glacial acetic acid per 100mL. The mobile flow rate was 0.5 mL/min and the injection volume was 3 µL.

4.2 Lipid class separation II – characterization of complex GSL

The isolation and purification of GSL were carried out using a micro method according to Barone *et al.* [55]. Despite this procedure is rather laborious and time-consuming, it has been shown to be particularly advantageous for less abundant complex GSL for which efficient isolation by traditional methods has not yet been reported. In total, 24 paired tissue samples were collected from 12 PDAC patients, pooled (tumor vs. adjacent normal tissues), and used for extraction. In addition, enzymatic digestion using recombinant endoglycoceramidase II (rEGCase II) from *Rhodococcus spp.* was used to release glycans from neutral GSL fractions in order to enhance the capability of isomeric separation. Analyses were carried out using an Accela 600 binary pump (Thermo) coupled with LTQ XL linear quadrupole ion trap mass spectrometer (Thermo). The separation of GSL-derived glycans and acidic GSL was achieved on porous graphitic carbon (PGC) and silica-based capillary column

packed in-house. The MS^2 patterns of GSL-derived glycans investigated in negative ion mode allowed clear distinction of isomers and deduction of glycan sequence and linkages positions via typical B- and C-type fragment ions and specific diagnostic fragments (D-type ions).

4.3 Plasma lipid profiles of three types of cancer

A modified Folch procedure was employed for the lipid extraction. Human serum (25 µL) and the mixture of IS (17.5 µL) were homogenized in 3 mL of chloroform/methanol (2:1, *v/v*) for 10 min in an ultrasonic bath (40 °C). When the samples reached ambient temperature, 600 µL of water was added, and the mixture was vortexed for 1 min. After 3 min of centrifugation (3000 rpm), the aqueous layer was removed, and the organic layer was evaporated under a gentle stream of nitrogen. The residue was dissolved in a mixture of 500 µL of chloroform/2-propanol (1:1, *v/v*), carefully vortexed and filtered through 0.2 µm syringe filter. The extract was diluted 1:20 with the mixture of hexane/2-propanol/chloroform (7:1.5:1.5, *v/v/v*) for UHPSFC/MS analysis and 1:8 with chloroform/methanol/2-propanol (1:2:4, *v/v/v*) mixture containing 7.5 mM of ammonium acetate and 1% of acetic acid for shotgun MS analysis. UHPSFC/MS measurements were carried out on an Acquity Ultra Performance Convergence Chromatography (UPC2) system hyphenated to the hybrid quadrupole traveling wave ion mobility time-of-flight mass spectrometer Synapt G2-Si from Waters using the commercial interface kit (Waters, Milford, MA, USA). UHPSFC analyses were measured on the Viridis BEH column (100 × 3 mm, 1.7 µm) using a linear gradient with supercritical CO_2 and a modifier, MeOH with 30 mM ammonium acetate and 1% water: 0 min–1% modifier, 5 min–51% modifier, 6.5 min–51% modifier, 6.8 min–1% modifier. The total run time including the equilibration was 7.5 min. The flow was 1.9 mL/min and the injection volume was 1 µL.

5 Results and discussion

5.1 Comprehensive characterization of simple GSL and other lipids in human plasma using HILIC-ESI/MS^2

Glycosphingolipids (GSL) are amphipathic and extremely diverse glycolipids covering a wide range of polarities, mainly from the polar to ionic region. Several studies have shown the association between alterations of GSL and onset of various human diseases including cancer. Therefore, one of the major attention has been devoted to the characterization of GSL and their role in progression and development of various pathophysiological processes. Given that GSL are typically low abundant in biofluids compared to other lipids and also suffer from low ionization efficiency, especially those heavily glycosylated, their isolation, detection, and complex structural analysis in biological samples poses a challenging task. In total, 154 GSL species within 8 subclasses (*i.e.,* GlcCer, GalCer, LacCer, Gb_3Cer, Gb_4Cer, SHexCer, $SHex_2Cer$, and GM_3) and 77 phospholipids from 4 subclasses (*i.e.,* PI, LPI, PE, LPE) were explicitly identified in human plasma using hydrophilic interaction liquid chromatography electrospray ionization tandem mass spectrometry (HILIC-ESI/MS^2), **Fig. 4**. Few other compounds, such as FA, Cer, PC, LPC, and SM were also detected. The identification and in-depth structural elucidation of individual lipid species were based on their retention times, *m/z* of precursor and product ions, and monitoring specific MS^2/MS^3 fragmentation patterns, such as glycan sequence and composition of the ceramide part of the GSL molecule in positive and negative ion modes.

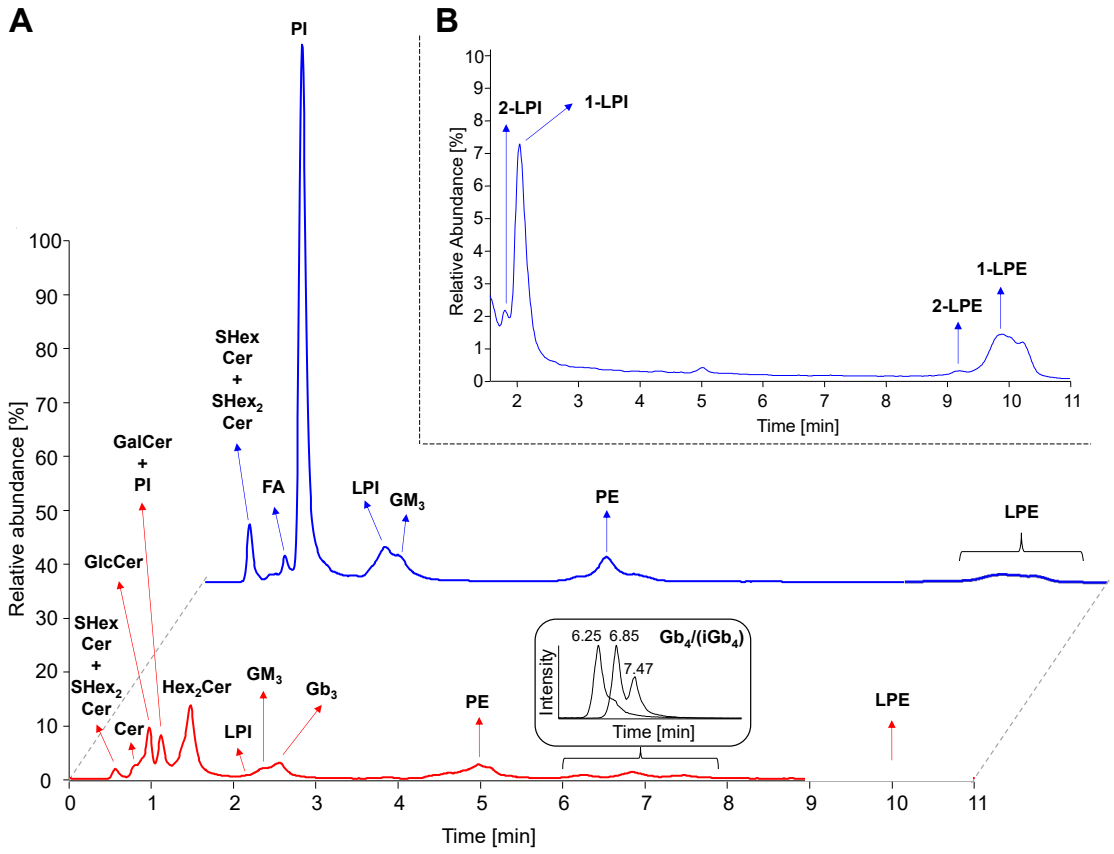

Fig. 4: Hydrophilic interaction liquid chromatography coupled to electrospray ionization mass spectrometry (HILIC-ESI/MS) chromatograms. (**A**) Reconstructed ion current (RIC) chromatograms of detected lipid subclasses (*m/z* 50–1500) in human plasma using both positive (bottom red line) and negative (upper blue line) polarity modes. (**B**) Chromatogram illustrating separation of *sn*-1 (i.e., 1-LPI and 1-LPE) and *sn*-2 (i.e., 2-LPI and 2-LPE) regioisomers of lysophospholipids.

Furthermore, the lipid profile of human plasma (**Fig. 5**) was generated upon identification. The lipid profiling revealed that the most abundant GSL species within particular subclasses were composed of ceramide 18:1;O2/16:0, respectively 34:1;O2, alongside numerous other usually low abundant GSL species. The only exception was sulfatide SHexCer, dominated by hydroxylated ceramide with the composition 18:1;O2/16:0;O, respectively 34:1;O3. It was also found that the majority of identified lipid species consisted of 18:1;O2 sphingosine. In addition, the extraction recovery at a medium concentration level using 1–2 IS per each lipid subclass (*i.e.,* exogenous and deuterated) was performed in triplicates. The purpose of this step was to illustrate that the developed and optimized method effectively extracts the GSL of interest and may be further used for future studies in biological samples. The extraction recovery of GSL ranged from 80.8 to 101.7% with relative standard deviation (RSD) varying from 4.0 to 12.5%. Most of the exogenous IS also fulfilled the requirement (*i.e.,* RSD ≤ 15%), except GlcCer (RSD 18.8%) and GM3 (RSD 17.0 %). Consequently, the developed HILIC-ESI-MS^2 method can be a useful tool for GSL profiling and may be used for further biological research of GSL in the biological samples.

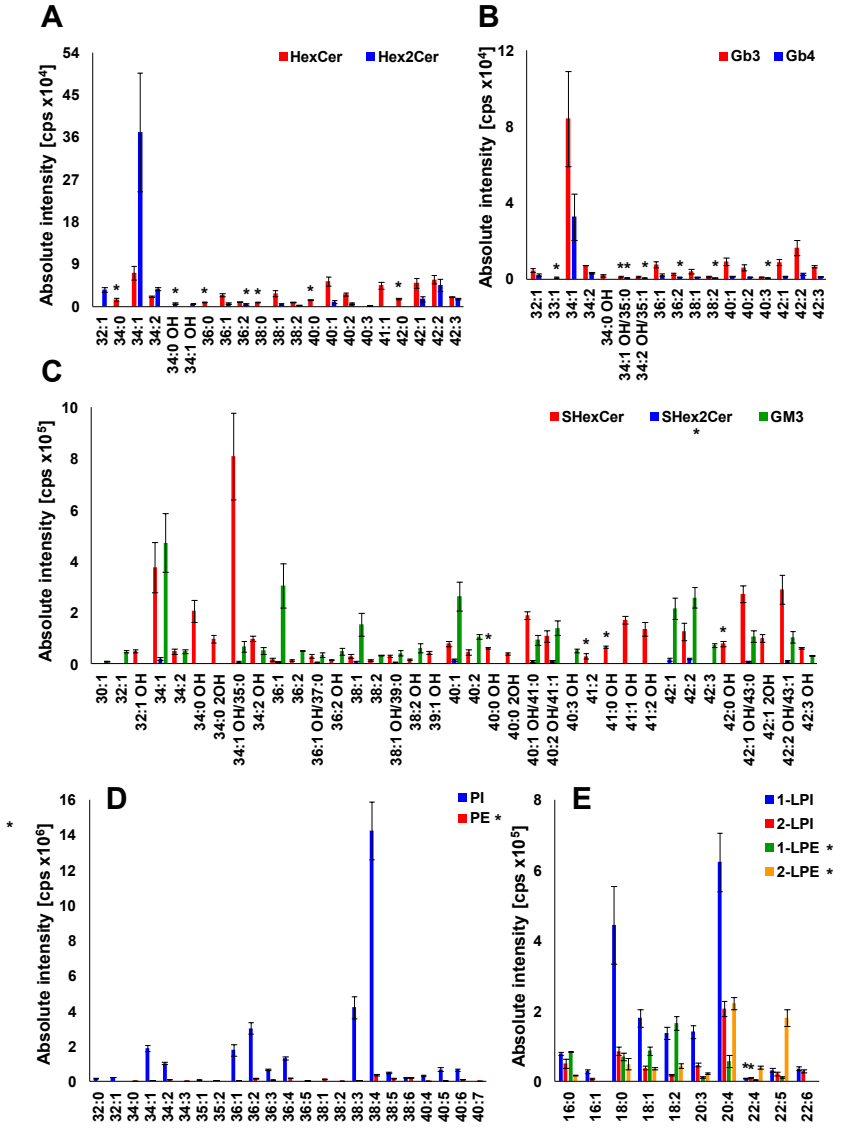

Fig. 5: Profile of human plasma lipids. (**A**) mono- and dihexosylceramides, (**B**) globotriaosyl- and globotetraosylceramides, (C) sulfatides and gangliosides, (**D**) phospholipids, and (**E**) lysophospholipids. Bar graphs represent the mean intensities (based on 3 replicates) as a function of sum composition with error bars corresponding to relative standard deviation (RSD). Lipid species labeled by an asterisk were not confirmed by MS/MS experiments.

5.2 Comprehensive characterization of complex GSL in human pancreatic cancer tissues using HPLC-ESI/MS^2

Plasma and/or serum are not the only biological samples suitable for lipidomic analysis. Lipid profiling can also be performed in other biological matrices, such as urine, saliva, cerebrospinal fluid, and notably tissues. A vast number of GSL have been shown to be implicated in various diseases including cancer. However, most of these studies are restricted only to simple GSL, such as GlcCer, GalCer, LacCer, and Gb_3Cer, together with several gangliosides and sulfatides, while altered complex GSL are shown only in a few studies. Consequently, special attention is devoted to the isolation and purification of complex GSL from tumors and adjacent normal tissues of PDAC patients, since the pancreas is one of the organs in which substantial amounts of ABH and Lewis antigens are present in epithelial cells of mainly pancreatic ducts. In addition, ABH antigens are suggested to be related to the tumorigenesis of the pancreas [56]. The major neutral GSL identified were GSL with 4 to 7 monosaccharide units bearing terminal blood group A, B, H, Le^a, Le^x, Le^b, Le^y, P1, and PX2 determinants alongside globo- (Gb_3Cer and Gb_4Cer), and neolacto-series GSL (nLc_4Cer and nLc_6Cer) (**Fig. 6**). Moreover, sulfatides and GM3 gangliosides were predominant acidic GSL together with minor sialyl-nLc_4Cer/nLc_6Cer and sialyl-Le^a/Le^x. On the contrary, the analysis of neutral GSL using the HILIC column provided a limited number of reliably identified GSL species due to sensitivity issues. Furthermore, the double peak formation resulting probably from the existence of both α and β anomer of Glc at the reducing end was observed in most GSL subclasses, with the identical composition confirmed by MS^2 experiments.

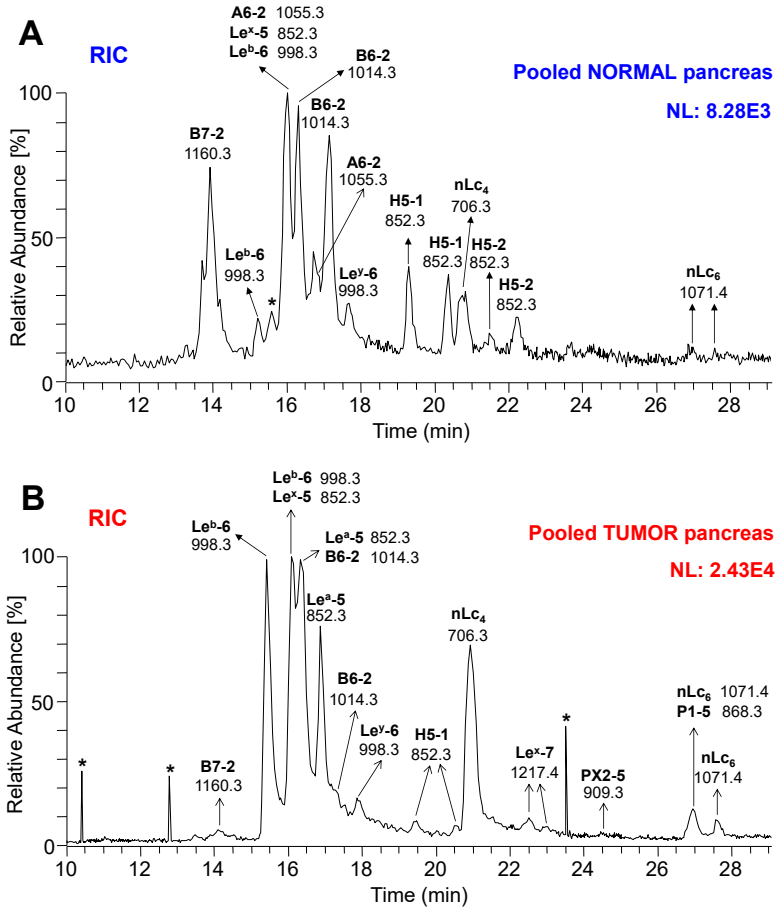

Fig. 6: Reconstructed ion current (RIC) chromatogram from LC/ESI-MS (negative ion mode at m/z 500–1300, retention time 10–29 min) of the neutral glycosphingolipid (GSL) fraction obtained from pooled normal **(A)** and tumor **(B)** pancreatic tissues depicting all identified and confirmed GSL subclasses.

TLC with anisaldehyde (**Fig. 7**) and resorcinol (**Fig. 8**) staining together with carbohydrate-recognizing binding assays using antibodies (*i.e.,* anti-Le^a, anti-Le^b, anti-A, anti-Neu5Acα3-nLc4, anti-Neu5Acα3-Lc4, anti-sLe^a, anti-sLe^x), lectins (*i.e., Erythrina crystagalli, Groffionia simplicifolia* IB4), and bacteria (*i.e.,* ^{35}S-labeled P-fimbriated *E. coli* strain 291-15) were tested to substantiate the data obtained from LC-ESI/MS^2.

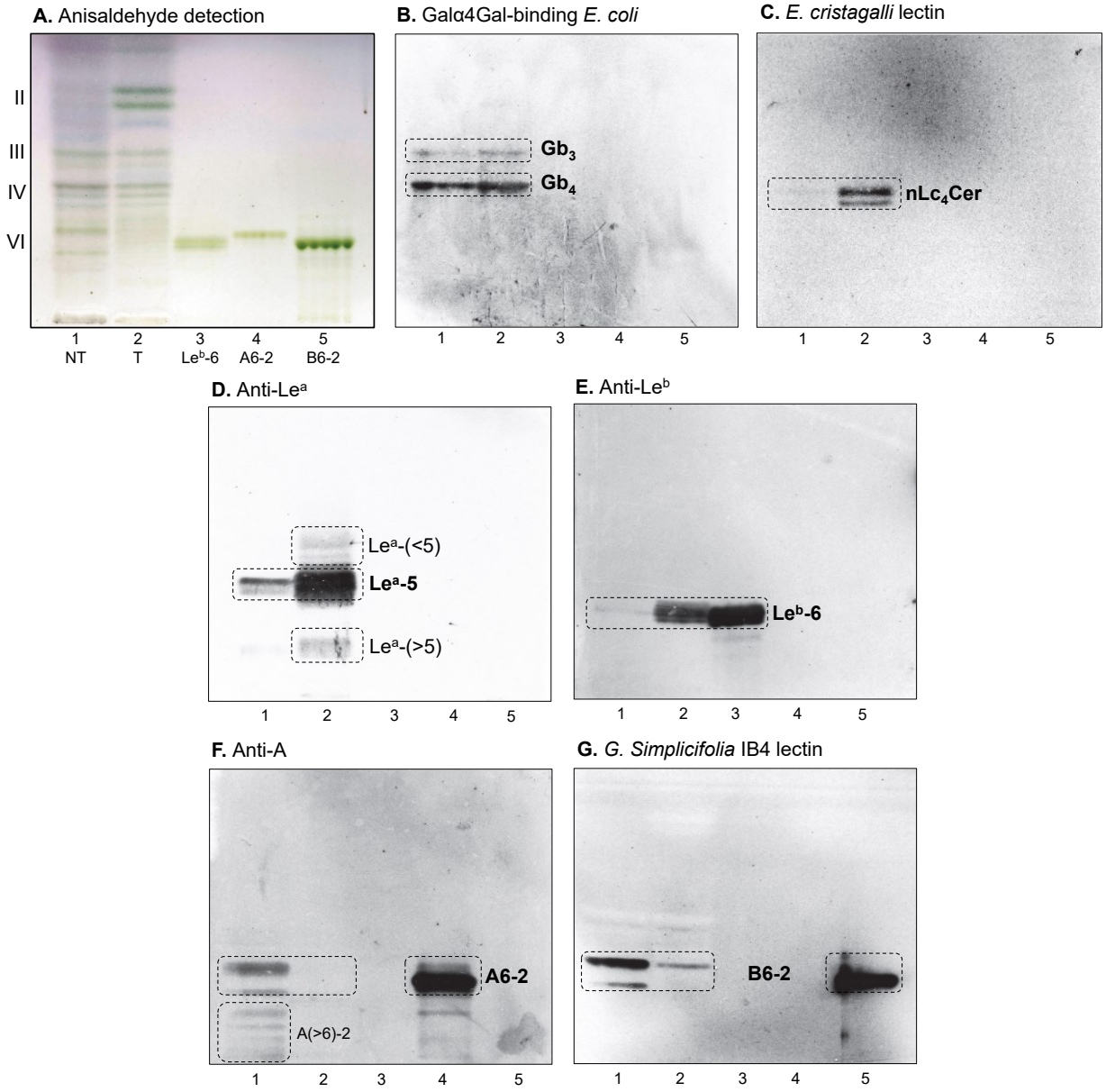

Fig. 7: Thin-layer chromatogram with anisaldehyde detection (A) and autoradiograms obtained by binding Galα4Gal-recognizing P-fimbriated *Escherichia coli* strain 291-15 (B), Galβ4GlcNAc-recognizing *Erythrina cristagalli* lectin (C), monoclonal antibodies directed against the blood group Le^a (D), Le^b (E), and A (F) determinants, and terminal Galα-recognizing *Griffonia simplicifolia* IB4 lectin (G). Lanes: lane 1, N-GSL fraction of pooled normal pancreatic tissue (NT), 40 µg; lane 2, N-GSL fraction of pooled pancreatic ductal adenocarcinoma tissue (T), 40 µg; lane 3, reference Leb-6, 4 µg; lane 4, reference A6-2, 4 µg; lane 5, reference B6-2, 4 µg. The Roman numbers to the left of chart A denote the number of carbohydrate units.

TLC staining revealed that major bands migrated in the regions with 1 to 4 monosaccharide units along with some minor slow-migrating compounds with 5 to 7 monosaccharide units. The outcomes of binding assays clearly illustrated the differences in GSL expression between tumor and adjacent normal pancreatic tissues in line with the LC-ESI/MS^2.

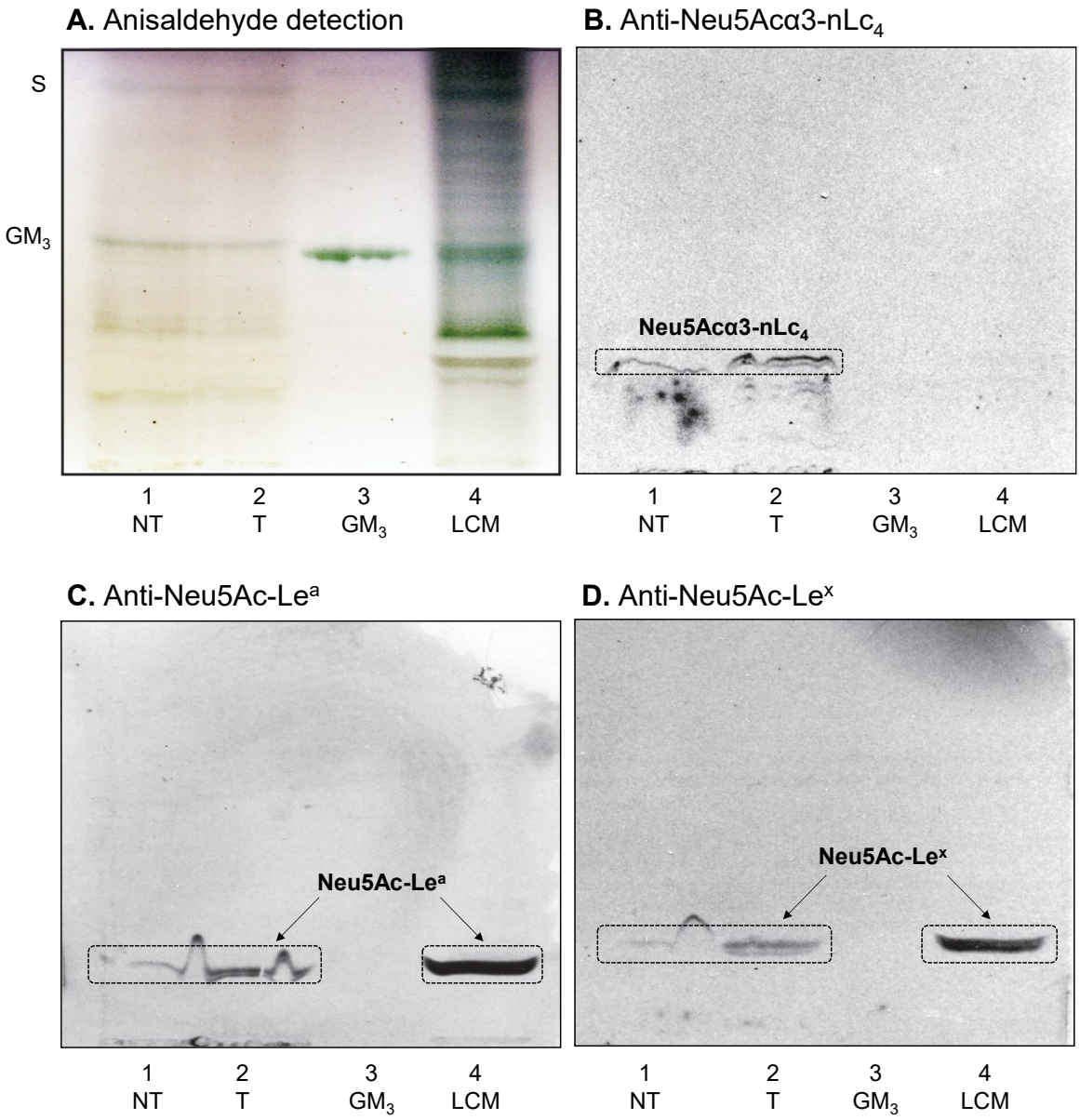

Fig. 8: Thin-layer chromatogram with anisaldehyde detection (A) and autoradiograms obtained by binding of monoclonal antibodies directed against the Neu5Acα3-nLc4 (B), Neu5Ac-Lea (C), and Neu5Ac-Lex (D) determinants. Lanes: lane 1, A-GSL fraction of pooled normal pancreatic tissue (NT), 40 µg; lane 2, A-GSL fraction of pooled pancreatic ductal adenocarcinoma tissue (T), 40 µg; lane 3, reference Neu5Ac-GM3, 4 µg; lane 4, reference A-GSL fraction of lung cancer metastasis (LCM), 40 µg. The designation S to the left of the chart A indicates the migration level of SGalCer and GM3 indicates the migration level of the Neu5Ac-GM3 gangliosides.

Glycan profiling of tumor and adjacent normal pancreatic tissues revealed differences in the region from 4 to 7 sugar units, and in type 1/2 core chains. Specifically, tumor pancreatic tissues were dominated by GSL with blood group Le^a and Le^b determinants (*i.e.,* type 1 chain) alongside nLc_4Cer, while the predominant GSL of adjacent normal pancreatic tissues were GSL with blood group A and B determinants (*i.e.,* type 2 chain). Other glycans including GSL with blood group Le^x, Le^y, H determinants, and nLc_6Cer were found in both tissues, while P1 and PX2 pentaosylceramides along with Le^y heptaosylceramides were minor components of tumor pancreatic tissues. Additionally, binding assays confirmed the presence of Gb_3Cer and Gb_4Cer, although they were not identified by LC-ESI/MS^2. In the case of acid GSL, the major GSL were sulfatides and GM3 gangliosides with 34:1;O2 and 34:1;O3 ceramides together

with minor s-nLc$_4$Cer/nLc$_6$Cer. Sialylated GSL, such as sLe^a (*i.e.,* CA 19-9 biomarker) and sLe^x, were determined by binding assays despite not being identified by LC-ESI/MS^2. Modifications of expression of ABH and Lewis blood group-related antigens are characteristic features of various carcinomas. The major changes in the glycosylation in most human cancers occur on type 2 chain, which is also the major carrier for the blood group ABH determinant of human red blood cells [57,58]. These changes are inevitably connected with the dysregulation of glycosyltransferases, namely fucosyltransferases (FUTs), which are responsible for the formation of Lewis blood group determinants. Namely, FUT1 has been shown to preferentially glycosylate type 2 chain. Conversely, FUT2 and FUT3 prioritize type 1 chain. This is in line with the observed predominance of fucosylated type 1 chain GSL (*i.e.,* Le^a-5 and Le^b-6) in tumor tissues and dominance of GSL with blood group A and B determinants (*i.e.,* A6-2, B6-2, and B7-2) on type 2 chain in adjacent normal tissues in this study. Therefore, the overexpression of Lewis blood group antigens in PDAC may be associated with the upregulation of FUT2 and/or FUT3. In contrast, the accumulation of nLc$_4$Cer along with the absence of blood group A and B associated GSL (*i.e.,* type 2 chain) may be in line with the downregulation of FUT1, the enzyme responsible for the formation of the H type 2 GSL, and the precursor of blood group A and B determinants on type 2 chain. The extensive investigation of complex GSL in human pancreatic cancer extends the coverage of GSL that are not routinely analyzed by traditional lipidomic methods. Furthermore, it provides an important platform for further studies of GSL alterations, such as glycosylation, sialylation, and/or fucosylation that are an integral part of many pathophysiological processes. It could also contribute to the development of new biomarkers and therapeutic approaches. The future perspective is to simplify the isolation protocol and to incorporate complex GSL into the body fluids-based screening methods of PDAC and possibly other cancers. However, future studies should clarify these results and investigate whether these differences translate into GSL profiles between patients and healthy subjects.

5.3 Lipid profiles of kidney, breast and prostate cancer patients differ from healthy controls

Another study comparing the plasma lipid profiles of healthy volunteers and patients with kidney, breast, and prostate cancer was carried out using UHPSFC-MS and DI-MS with the aim to differentiate cancer patients from healthy controls. In total, 289 samples of cancer patients (*i.e.,* 119 kidney, 103 breast, and 67 prostate) and 192 samples of healthy volunteers were collected and analyzed. Samples were divided into training set (ca 75%) and validation set (ca 25%) in a similar percentage representation of individual types of samples. The analysis resulted in the quantitation of 138 lipids from GL, GP, and SP subclasses, from which 91 lipids were selected for multivariate data analysis. Then, the number of lipid species was further reduced to seven lipids by applying additional statistical criteria, such as fold change, *p*-value, VIP value, and Bonferroni correction. Models of cancer prediction were characterized by high sensitivity, specificity, and accuracy. Statistically, the most dysregulated lipids were CE 16:0, Cer 42:1;O2, LPC 18:2, PC 36:2, PC 36:3, SM 32:1;O2, and SM 41:1;O2, which may represent potential biomarkers to differentiate kidney, breast, and prostate cancer from healthy volunteers based on human plasma profiling. In conclusion, the data indicated the potential of lipid profiling with the use of multivariate data analysis as a diagnostic tool for all three studied types of cancer.

5.4 Recent advances, challenges, and future directions in the mass spectrometry analysis of glycosphingolipids in biological samples

The last part of my work is a review article. The review first discusses technological advances in GSL analysis, such as the replacement of conventional HPLC columns with sub-2-µm particles, offering enhanced separation efficiency and high-throughput analysis, the use of 2D SFC/RPLC-MS and UHPSFC-MS for improved lipidomic coverage, selectivity, and sensitivity, or implementation of IM technologies, adding a fourth dimension of separation, together with recent innovations (*e.g.,* PASEF, SLIM) enhancing the separation of isomers. These advancements contribute to a comprehensive understanding of GSL in a biological context. The next section addresses the challenges in the GSL analysis, focusing on sample pre-treatment, extraction and purification strategies that are crucial to maintain the integrity of the sample. The need for innovative isolation protocols for efficient GSL extraction from complex matrices is emphasized as the well-established protocols are not sufficient. The challenges, such as achieving rapid and uniform extraction, along with the complete removal of interfering substances (*e.g.,* using alkaline hydrolysis, ZrO_2/TiO_2-based SPE, and silica-based or weak-anion exchange chromatography) that persist are highlighted. Then the review is focused on issues related to the separation of isomers, as the baseline separation of GSL isomers is still challenging even when applying IM with innovative technologies. Various derivatization techniques, such as permethylation, isobaric labelling, or benzoyl derivatization, designed to increase the ionization efficiency of GSL and/or to aid in the structural elucidation by introducing specific fragment ions are discussed as well. The section addressing quantitative analysis and validation emphasizes the limited availability of suitable ISs for quantitation and suggests alternative strategies, such as chemoenzymatic synthesis or *in vivo* stable isotope labeling, a promising approaches to reliable and accurate quantitation addressing the lack of ISs for GSL. The importance of method validation and harmonized protocols or guidelines is also discussed together with the absence of well-defined and certified reference materials that complicate the inter-laboratory comparisons. Furthermore, the development of novel, comprehensive, and open-source bioinformatics tools is crucial, as lipidomic analyses generate large datasets, to support lipidomics' potential for clinical diagnostics and understanding the roles of lipids in various pathophysiological processes. Although lipidomics is promising for monitoring diseases, including cancer, through non-invasive biomarkers obtained from body fluids, there are not so many GSL biomarkers that are used as diagnostic or prognostic markers. Moreover, the lack of extensive population-based clinical trials hinders the translation into clinical lipidomics. Consequently, future directions should focus on the efficient isolation of more complex and minor GSL, increased ionization efficiency, enhanced separation of GSL isomers, and development of stable-isotope labelling method for accurate absolute quantitation. In conclusion, continuous efforts are crucial to address these limitations to fully reveal the potential of glycosphingolipidomics for clinical translation as comprehensive high-throughput GSL profiling in biological samples remains very challenging.

Conclusions

This dissertation deals with the development and optimization of methods for detailed structural characterization and profiling of GSL, GSL-derived glycans, and other lipids in biological samples using HPLC-ESI-MS/MS. The theoretical part provides a detailed and comprehensive review describing the structure, classification, nomenclature, and biological functions of GSL together with their associations with various diseases. Various sample preparation methods and analytical methods, with the emphasis on the qualitative and quantitative analysis of GSL and other lipids, are discussed in detail as well. The experimental part is devoted to the development and optimization of new liquid-liquid extraction followed by solid-phase extraction using the HILIC-ESI-MS/MS method. This method enabled the identification and characterization of 154 simple GSL species within 7 lipid subclasses in human plasma together with 77 phospholipids and several ceramides and fatty acids. The HPLC-ESI-MS/MS method using a PGC column capable of separating structural isomers was applied for the analysis of GSL-derived glycans extracted and isolated from human pancreatic cancer tissues and adjacent normal pancreatic tissues. This method primarily allowed the profiling of more complex GSL. The analysis also revealed striking differences in glycosylation, mainly fucosylation and sialylation, between the tumor and adjacent normal tissues. It was found that the type 1 core chain represented by GSL carrying Le^a and Le^b determinants along with nLc_4Cer predominated in tumor tissues, while the type 2 core chain represented by GSL with blood group A and B determinants were preferentially found in adjacent normal tissues. These findings were also supported by chromatogram binding assays using specific antibodies, bacteria, and lectins. UHPSFC-MS method has been applied for the differentiation of patients with kidney, breast and prostate cancer from healthy controls based on the quantitative lipid profiling, where lipids were extracted from human plasma. Finally, recent advances, current challenges, and future directions in the analysis of GSL in biological samples were reviewed. The related works demonstrate that the coupling of chromatographic techniques with mass spectrometry for the analysis of GSL has recently greatly advanced. However, there are still a few issues complicating the reliable identification and quantitation, namely (1) variability in extraction protocols that do not effectively extract a wide range of GSL, (2) lack of appropriate ISs for accurate quantitation, and (3) inability to differentiate isomers and/or isobars. Future directions should also focus on the development of novel and unique extraction protocols capable of isolating simple as well as more complex and even minor GSL subclasses together with the development of suitable methods for the synthesis of ISs that could compensate for the lack of ISs. These improvements are necessary to improve the possibilities of finding suitable biomarkers that could provide valuable information and enable the detection of various diseases in the early stages and assure timely treatment to improve clinical outcomes.

List of References

[1] A.H. Merrill, M.D. Wang, M. Park, M.C. Sullards, (Glyco)sphingolipidology: an amazing challenge and opportunity for systems biology, Trends Biochem. Sci. 32 (2007) 457–468. https://doi.org/10.1016/j.tibs.2007.09.004.

[2] X. Zhang, F.L. Kiechle, Review: Glycosphingolipids in Health and Disease, Ann. Clin. Lab. Sci. 34 (2004) 3–13.

[3] H. -J Senn, M. Orth, E. Fitzke, H. Wieland, W. Gerok, Gangliosides in normal human serum: Concentration, pattern and transport by lipoproteins, Eur. J. Biochem. 181 (1989) 657–662. https://doi.org/10.1111/j.1432-1033.1989.tb14773.x.

[4] S. Hakomori, Glycosphingolipids, Sci. Am. 254 (1986) 44–53. https://doi.org/10.1038/scientificamerican0586-44.

[5] X. Han, Lipidomics: Comprehensive Mass Spectrometry of Lipids, 1st ed., John Wiley & Sons, Inc., Hoboken, NJ, USA, 2016. https://doi.org/10.1002/9781119085263.

[6] R.L.T. Schnaar, T. Kinoshita, Glycosphingolipids, in: Essentials Glycobiol. 3rd Ed., 2017: pp. 1–11.

[7] G. D'Angelo, S. Capasso, L. Sticco, D. Russo, Glycosphingolipids: Synthesis and functions, FEBS J. 280 (2013) 6338–6353. https://doi.org/10.1111/febs.12559.

[8] K. Furukawa, Y. Ohmi, Y. Ohkawa, R.H. Bhuiyan, P. Zhang, O. Tajima, N. Hashimoto, K. Hamamura, K. Furukawa, New era of research on cancer-associated glycosphingolipids, Cancer Sci. 110 (2019) 1544–1551. https://doi.org/10.1111/cas.14005.

[9] J.A. Low, B. Magnuson, B. Tsai, M.J. Imperiale, Identification of Gangliosides GD1b and GT1b as Receptors for BK Virus, J. Virol. 80 (2006) 1361–1366. https://doi.org/10.1128/jvi.80.3.1361-1366.2006.

[10] B. Tsai, J.M. Gilbert, T. Stehle, W. Lencer, T.L. Benjamin, T.A. Rapoport, Gangliosides are receptors for murine polyoma virus and SV40, EMBO J. 22 (2003) 4346–4355. https://doi.org/10.1093/emboj/cdg439.

[11] M. Kitamura, K. Takamiya, S. Aizawa, K. Furukawa, K. Furukawa, Gangliosides are the binding substances in neural cells for tetanus and botulinum toxins in mice, Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 1441 (1999) 1–3. https://doi.org/10.1016/S1388-1981(99)00140-7.

[12] C.A. Lingwood, H. Law, S. Richardson, M. Petric, J.L. Brunton, S. De Grandis, M. Karmali, Glycolipid binding of purified and recombinant Escherichia coli produced verotoxin in vitro., J. Biol. Chem. 262 (1987) 8834–8839. https://doi.org/10.1016/s0021-9258(18)47490-x.

[13] S.I. Hakomori, The glycosynapse, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 225–232. https://doi.org/10.1073/pnas.012540899.

[14] R.L. Schnaar, Glycosphingolipids in cell surface recognition, Glycobiology. 1 (1991) 477–485. https://doi.org/10.1093/glycob/1.5.477.

[15] S. Hakomori, Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions, J. Biol. Chem. 265 (1990) 18713–18716. https://doi.org/10.1016/s0021-9258(17)30565-3.

[16] S. Hakomori, K. Handa, K. Iwabuchi, S. Yamamura, A. Prinetti, Glyco-Forum section, 8 (1998).

[17] K. Iwabuchi, K. Handa, S. Hakomori, Separation of " Glycosphingolipid Signaling Domain " from Caveolin-containing Membrane Fraction in Mouse Melanoma B16

Cells and Its Role in Cell Adhesion Coupled with Signaling *, 273 (1998) 33766–33773.
[18] A.J. Yates, A. Rampersaud, Sphingolipids as receptor modulators: An overview, Ann. N. Y. Acad. Sci. 845 (1998) 57–71. https://doi.org/10.1111/j.1749-6632.1998.tb09662.x.
[19] P.J. Meikle, G. Wong, C.K. Barlow, B.A. Kingwell, Lipidomics: Potential role in risk prediction and therapeutic monitoring for diabetes and cardiovascular disease, Pharmacol. Ther. 143 (2014) 12–23. https://doi.org/10.1016/j.pharmthera.2014.02.001.
[20] E. Miyoshi, K. Moriwaki, T. Nakagawa, Biological function of fucosylation in cancer biology, J. Biochem. 143 (2008) 725–729. https://doi.org/10.1093/jb/mvn011.
[21] C.P. Delannoy, Y. Rombouts, S. Groux-Degroote, S. Holst, B. Coddeville, A. Harduin-Lepers, M. Wuhrer, E. Elass-Rochard, Y. Guérardel, Glycosylation Changes Triggered by the Differentiation of Monocytic THP-1 Cell Line into Macrophages, J. Proteome Res. 16 (2017) 156–169. https://doi.org/10.1021/acs.jproteome.6b00161.
[22] N. Schömel, G. Geisslinger, M.S. Wegner, Influence of glycosphingolipids on cancer cell energy metabolism, Prog. Lipid Res. 79 (2020) 101050. https://doi.org/10.1016/j.plipres.2020.101050.
[23] L. Cooling, Blood groups in infection and host susceptibility, Clin. Microbiol. Rev. 28 (2015) 801–870. https://doi.org/10.1128/CMR.00109-14.
[24] G. Daniels, Blood groups on red cells, platelets and neutrophils, in: Blood Bone Marrow Pathol. Expert Consult, Second Edi, Elsevier Ltd, 2011: pp. 599–617. https://doi.org/10.1016/B978-0-7020-3147-2.00037-7.
[25] R.K. Saini, P. Prasad, X. Shang, Y.S. Keum, Advances in lipid extraction methods—a review, Int. J. Mol. Sci. 22 (2021) 1–19. https://doi.org/10.3390/ijms222413643.
[26] J. Folch, M. Lees, G.H. Sloane Stanley, A simple method for the isolation and purification of total lipides from animal tissues., J. Biol. Chem. 226 (1957) 497–509. https://doi.org/10.1016/s0021-9258(18)64849-5.
[27] W.J. Bligh, E.G. and Dyer, A Rapid Method of Total Lipid Extraction and Purification, Can. J. Biochem. Physiol. 37 (1959) 911–917. https://doi.org/10.1139/o59-099.
[28] D.F. Smith, P.A. Prieto, Special Considerations for Glycolipids and Their Purification, Curr. Protoc. Mol. Biol. 22 (1993) 1–13. https://doi.org/10.1002/0471142727.mb1703s22.
[29] R.C. Barrientos, Q. Zhang, Recent advances in the mass spectrometric analysis of glycosphingolipidome – A review, Anal. Chim. Acta. 1132 (2020) 134–155. https://doi.org/10.1016/j.aca.2020.05.051.
[30] V. Matyash, G. Liebisch, T. V. Kurzchalia, A. Shevchenko, D. Schwudke, Lipid extraction by methyl-terf-butyl ether for high-throughput lipidomics, J. Lipid Res. 49 (2008) 1137–1146. https://doi.org/10.1194/jlr.D700041-JLR200.
[31] L. Löfgren, M. Ståhlman, G.B. Forsberg, S. Saarinen, R. Nilsson, G.I. Hansson, The BUME method: A novel automated chloroform-free 96-well total lipid extraction method for blood plasma, J. Lipid Res. 53 (2012) 1690–1700. https://doi.org/10.1194/jlr.D023036.
[32] L. Löfgren, G.B. Forsberg, M. Ståhlman, The BUME method: A new rapid and simple chloroform-free method for total lipid extraction of animal tissue, Sci. Rep. 6 (2016). https://doi.org/10.1038/srep27688.
[33] Z.H. Alshehry, C.K. Barlow, J.M. Weir, Y. Zhou, M.J. McConville, P.J. Meikle, An

efficient single phase method for the extraction of plasma lipids, Metabolites. 5 (2015) 389–403. https://doi.org/10.3390/metabo5020389.
[34] G. Vale, S.A. Martin, M.A. Mitsche, B.M. Thompson, K.M. Eckert, J.G. McDonald, Three-phase liquid extraction: A simple and fast method for lipidomic workflows, J. Lipid Res. 60 (2019) 694–706. https://doi.org/10.1194/jlr.D090795.
[35] C.S. Ejsing, J.L. Sampaio, V. Surendranath, E. Duchoslav, K. Ekroos, R.W. Klemm, K. Simons, A. Shevchenko, Global analysis of the yeast lipidome by quantitative shotgun mass spectrometry, Proc. Natl. Acad. Sci. U. S. A. 106 (2009) 2136–2141. https://doi.org/10.1073/pnas.0811700106.
[36] M.W.K. Wong, N. Braidy, R. Pickford, P.S. Sachdev, A. Poljak, Comparison of single phase and biphasic extraction protocols for lipidomic studies using human plasma, Front. Neurol. 10 (2019) 1–11. https://doi.org/10.3389/fneur.2019.00879.
[37] C. Hu, C. Wang, L. He, X. Han, Novel strategies for enhancing shotgun lipidomics for comprehensive analysis of cellular lipidomes, TrAC - Trends Anal. Chem. 120 (2019). https://doi.org/10.1016/j.trac.2018.11.028.
[38] M. Holčapek, G. Liebisch, K. Ekroos, Lipidomic Analysis, Anal. Chem. 90 (2018) 4249–4257. https://doi.org/10.1021/acs.analchem.7b05395.
[39] M. Ståhlman, C.S. Ejsing, K. Tarasov, J. Perman, J. Borén, K. Ekroos, High-throughput shotgun lipidomics by quadrupole time-of-flight mass spectrometry, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 877 (2009) 2664–2672. https://doi.org/10.1016/j.jchromb.2009.02.037.
[40] X. Han, Multi-dimensional mass spectrometry-based shotgun lipidomics and the altered lipids at the mild cognitive impairment stage of Alzheimer's disease, Biochim. Biophys. Acta - Mol. Cell Biol. Lipids. 1801 (2010) 774–783. https://doi.org/10.1016/j.bbalip.2010.01.010.
[41] K. Yang, Z. Zhao, R.W. Gross, X. Han, Systematic analysis of choline-containing phospholipids using multi-dimensional mass spectrometry-based shotgun lipidomics, J. Chromatogr. B Anal. Technol. Biomed. Life Sci. 877 (2009) 2924–2936. https://doi.org/10.1016/j.jchromb.2009.01.016.
[42] N.E. Manicke, J.M. Wiseman, D.R. Ifa, R.G. Cooks, Desorption Electrospray Ionization (DESI) Mass Spectrometry and Tandem Mass Spectrometry (MS/MS) of Phospholipids and Sphingolipids: Ionization, Adduct Formation, and Fragmentation, J. Am. Soc. Mass Spectrom. 19 (2008) 531–543. https://doi.org/10.1016/j.jasms.2007.12.003.
[43] P. Nemes, A.S. Woods, A. Vertes, Simultaneous imaging of small metabolites and lipids in rat brain tissues at atmospheric pressure by laser ablation electrospray ionization mass spectrometry, Anal. Chem. 82 (2010) 982–988. https://doi.org/10.1021/ac902245p.
[44] A. Muck, T. Stelzner, U. Hübner, S. Christiansen, A. Svatoš, Lithographically patterned silicon nanowire arrays for matrix free LDI-TOF/MS analysis of lipids, Lab Chip. 10 (2010) 320–325. https://doi.org/10.1039/b913212k.
[45] Z. Wu, G.I. Bagarolo, S. Thoröe-Boveleth, J. Jankowski, "Lipidomics": Mass spectrometric and chemometric analyses of lipids, Adv. Drug Deliv. Rev. 159 (2020) 294–307. https://doi.org/10.1016/j.addr.2020.06.009.
[46] S.M. Camunas-Alberca, M. Moran-Garrido, J. Sáiz, A. Gil-de-la-Fuente, C. Barbas, A. Gradillas, Integrating the potential of ion mobility spectrometry-mass spectrometry in the separation and structural characterisation of lipid isomers, Front. Mol. Biosci. 10 (2023) 1–21. https://doi.org/10.3389/fmolb.2023.1112521.

[47] P. Ge, Y. Luo, H. Chen, J. Liu, H. Guo, C. Xu, J. Qu, G. Zhang, H. Chen, Application of Mass Spectrometry in Pancreatic Cancer Translational Research, Front. Oncol. 11 (2021) 1–16. https://doi.org/10.3389/fonc.2021.667427.
[48] C.C. Teo, W.P.K. Chong, E. Tan, N.B. Basri, Z.J. Low, Y.S. Ho, Advances in sample preparation and analytical techniques for lipidomics study of clinical samples, TrAC - Trends Anal. Chem. 66 (2015) 1–18. https://doi.org/10.1016/j.trac.2014.10.010.
[49] M. Li, Z. Zhou, H. Nie, Y. Bai, H. Liu, Recent advances of chromatography and mass spectrometry in lipidomics, Anal. Bioanal. Chem. 399 (2011) 243–249. https://doi.org/10.1007/s00216-010-4327-y.
[50] E. Torretta, C. Fania, M. Vasso, C. Gelfi, HPTLC-MALDI MS for (glyco)sphingolipid multiplexing in tissues and blood: A promising strategy for biomarker discovery and clinical applications, Electrophoresis. 37 (2016) 2036–2049. https://doi.org/10.1002/elps.201600094.
[51] J.I. Furukawa, S. Sakai, I. Yokota, K. Okada, H. Hanamatsu, T. Kobayashi, Y. Yoshida, K. Higashino, T. Tamura, Y. Igarashi, Y. Shinohara, Quantitative GSL-glycome analysis of human whole serum based on an EGCase digestion and glycoblotting method, J. Lipid Res. 56 (2015) 2399–2407. https://doi.org/10.1194/jlr.D062083.
[52] J.L. Norris, R.M. Caprioli, Analysis of tissue specimens by matrix-assisted laser desorption/ionization imaging mass spectrometry in biological and clinical research, Chem. Rev. 113 (2013) 2309–2342. https://doi.org/10.1021/cr3004295.
[53] S.R. Ellis, M.R.L. Paine, G.B. Eijkel, J.K. Pauling, P. Husen, M.W. Jervelund, M. Hermansson, C.S. Ejsing, R.M.A. Heeren, Automated, parallel mass spectrometry imaging and structural identification of lipids, Nat. Methods. 15 (2018) 515–518. https://doi.org/10.1038/s41592-018-0010-6.
[54] R. Jirásko, M. Holčapek, M. Khalikova, D. Vrána, V. Študent, Z. Prouzová, B. Melichar, MALDI Orbitrap Mass Spectrometry Profiling of Dysregulated Sulfoglycosphingolipids in Renal Cell Carcinoma Tissues, J. Am. Soc. Mass Spectrom. 28 (2017) 1562–1574. https://doi.org/10.1007/s13361-017-1644-9.
[55] A. Barone, J. Benktander, S. Teneberg, M.E. Breimer, Characterization of acid and non-acid glycosphingolipids of porcine heart valve cusps as potential immune targets in biological heart valve grafts, Xenotransplantation. 21 (2014) 510–522. https://doi.org/10.1111/xen.12123.
[56] R.-H. Li, L. Zhang, M.-Y. Wu, Tissue isoantigens A, B and H in primary carcinoma of the pancreas, Wourld J. Gastroenterol. 2 (1996) 241–242. https://doi.org/10.3748/wjg.v2.i4.241.
[57] H. Hattori, K. ichi Uemura, H. Ishihara, H. Ogata, Glycolipid of human pancreatic cancer; the appearance of neolacto-series (type 2 chain) glycolipid and the presence of incompatible blood group antigen in tumor tissues, Biochim. Biophys. Acta (BBA)/Lipids Lipid Metab. 1125 (1992) 21–27. https://doi.org/10.1016/0005-2760(92)90150-T.
[58] D. Wang, K. Madunić, T. Zhang, O.A. Mayboroda, G.S.M. Lageveen-Kammeijer, M. Wuhrer, High Diversity of Glycosphingolipid Glycans of Colorectal Cancer Cell Lines Reflects the Cellular Differentiation Phenotype, Mol. Cell. Proteomics. 21 (2022) 0–14. https://doi.org/10.1016/j.mcpro.2022.100239.

List of Students' Published Works

Submitted papers

1. **Hořejší K.,** and Holčapek M. Unraveling the complexity of glycosphingolipidome: The key role of mass spectrometry in the structural analysis of glycosphingolipids. *Anal. Bioanal. Chem.* (2024). (Q1; IF = 4.3; CIn = 0.93; submitted on June 17, 2024)

Published papers

1. **Hořejší K.,** Kolářová D., Jirásko R., and Holčapek M. Recent advances, challenges, and future direction in the mass spectrometry analysis of glycosphingolipids in biological samples. *Trac-Trends Anal. Chem.* **178** (2024) **117827**. doi: 10.1016/j.trac.2024.117827 (Q1(D1); IF = 13.1; CIn = 1.31, number of citation: 0)

2. **Hořejší K.,** Jin Ch., Vaňková Z., Jirásko R., Strouhal O., Melichar B., Teneberg S., and Holčapek M. Comprehensive characterization of complex glycosphingolipids in human pancreatic cancer tissues. *J. Biol. Chem.* **299** (2023) **102923**. doi: 10.1016/j.jbc.2023.102923 (Q2; IF = 4.8; CIn = 0.87; citations: 6)

3. Wolrab D., Jirásko R., Peterka O., Idkowiak J., Chocholoušková M., Vaňková Z., **Hořejší K.,** Brabcová I., Vrána D., Študentová H., Melichar B., and Holčapek M. Plasma lipidomic profiles of kidney, breast and prostate cancer patients differ from healthy controls. *Sci. Rep.* **11** (2021) **20322**. doi: 10.1038/s41598-021-99586-1 (Q2; IF = 4.6; CIn = 1.06; citations: 16)

4. **Hořejší K.,** Jirásko R., Chocholoušková M., Wolrab D., Kahoun D., and Holčapek M. Comprehensive Identification of Glycosphingolipids in Human Plasma Using Hydrophilic Interaction Liquid Chromatography–Electrospray Ionization Mass Spectrometry. *Metabolites.* **11** (2021) **140**. doi: 10.3390/metabo11030140 (Q2; IF = 4.1; CIn = 0.71; citations: 7)