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Mass Spectrometric Characterization of Sphingolipids and Glycosphingolipids in Biological Tissues

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Abstract

This doctoral thesis is deals on analysis of lipids in several complex samples using high-performance liquid chromatography coupled with mass spectrometry to obtain detailed information of the lipidome. The first part of this thesis aims to the development of a new method based on hydrophilic interaction liquid chromatography coupled to a negative-ion electrospray ionization high-resolution mass spectrometry used for the identification of sphingolipids mainly gangliosides and other polar lipids in biological samples. The identification of lipids was based on accurate *m/z* values of $[M-H]^-$ ions and fragment ions measured by high-resolution mass spectrometer. The next part of the thesis is focusing on optimization of the negative-ion hydrophilic liquid chromatography-electrospray ionization mass spectrometry method used for the quantitative analysis of ganglioside (GM3) and other polar lipid classes. The method was used for the quantitation of the studied lipids in kidney tumor and surrounding normal tissues on patients suffering by renal cell carcinoma. The raw data were statistically evaluated using multivariate data analysis methods.

Abstrakt

Tato disertační práce se zabývá analýzou lipidů v několika rozdílných vzorcích pomocí vysokoúčinné kapalinové chromatografie ve spojení s hmotnostní spektrometrií pro detailní informaci o lipidomu. První část této práce se zaměřuje na vývoj nové metody pro identifikaci sfingolipidů především gangliosidů a dalších polárních lipidů v biologických vzorcích. Metoda využívá kapalinovou chromatografii hydrofilních interakcí ve spojení s vysokorozlišujícím hmotnostním spektrometrem při použití elektrosprejové ionizace v negativním iontovém módu. Identifikace lipidů byla založena na základě správné *m/z* $[M-H]^-$ a fragmentových iontů. Druhá část této práce se zaměřuje na optimalizaci výše vyvinuté metody pro kvatitativní analýzu gangliosidů (GM3) a ostatních polárních lipidů. Metoda byla použita pro kvantitativní studii lipidů v nádorové tkáni ledvin a okolní nádorové tkáně ledvin u pacientů trpících karcinomem ledvin. Naměřená data byla statisticky vyhodnocena pomocí vícerozměrné statistické analýzy dat.

Keywords

Lipids, gangliosides, liquid chromatography, mass spektrometry.

Klíčová slova

Lipidy, gangliosidy, kapalinová chromatografie, hmotnostní spektrometrie.

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1. Introduction

1.1 Lipids

Lipids are defined as hydrophobic or amphipathic small molecules that originate entirely or in part by carbanion-based condensations of thioesters and/or by carbocation-based condensations of isoprene units [1, 2]. Lipids are molecules soluble in organic solvents and/or in water depending on the molecule of lipids. Lipids play significant role in physiological processes, such as interactions between cells of the immune system, in the cellular signaling, regulation of cell apoptosis, differentiation, and transformation [3-7]. The dysregulation of lipids is related to various human diseases, such as cancer, Alzheimer disease, and cardiovascular diseases [8].

Lipids are divided into eight major lipid categories (fatty acyls, glycerolipids, glycerophospholipids, sterol lipids, prenol lipids, saccharolipids, polyketides, and sphingolipids) according LIPID MAPS consortium [9]. Each group containing classes and subclasses of lipids. For example, the glycerophospholipids contains 21 classes and many subclasses, including glycerophosphocholines (PC), glycerophosphoethanolamines (PE), glycerophosphoserines (PS), glycerophosphoglycerols (PG), phosphatidylinositoles (PI), etc. The sphingolipids which they are important for this work comprises highly diverse lipid species. The fatty acyls of sphingolipids are linked via amide bond to a long chain base or sphingoid. The group of acidic sphingolipids contains gangliosides, which are sphingolipids containing the sphingoid base (ceramide) attached to mono- or polysialylated oligosaccharides. Gangliosides may have a different composition of sphingoid base, N-fatty acyls, and an oligosaccharide part, which results in a large complexity of natural gangliosides. Ceramide moiety can be attached to various sugars: one or more uncharged sugars, such as glucose (Glc) or galactose (Gal), with possible further attachment of other neutral sugars, such as N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), and fucose (Fuc); ionized functional groups, such as sulfate attached to uncharged sugars or sialic acid residues. Sialic acids (Sia) form a large group of neuraminic acid derivatives and may occur in various forms [10-12]. About 50 Sia types are known with N-acetylneuraminic acid (NeuAc), N-glycolyl neuraminic acid (NeuGc) and O-acetylated derivatives among which N-acetyl-9-O-acetylneuraminic acid is most common. Sialic acid is a trivial name used for all derivatives of neuraminic acid [4], where the most important is N-acetylneuraminic acid (NeuAc) and less common is N-glycolylneuraminic acid (NeuGc). NeuAc and NeuGc are structurally similar, but they differ significantly in their natural occurrence. N-acetylneuraminic acid is present in human unlike N-glycolylneuraminic acid, which is obtained only from the diet in a limited amount [4, 13, 14].

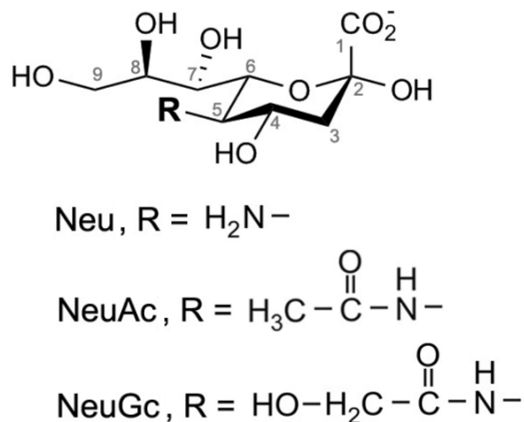

Figure 1: Sialic acids.

The nomenclature of ganglioside was published by Svennerholm [15] and later was approved by IUPAC [16]. In the shorthand notation system of gangliosides, the first letter G means ganglioside, the second letter indicates the number of sialic acids (M = mono, D = di, T = tri, and Q = tetra), then the number of neutral sugars is calculated as 5−n saccharide units, which may be followed by small letter a, b, or c defining the position of sialic acid(s). For example, GD1a (Figure 19), ganglioside (G) containing two Sia (D, 2x NeuAc), four neutral saccharides (n = 4, ie, 5-4 = 1) and a letter determines the position of Sia. The colon-separated numbers (e.g., 38:1) behind ganglioside abbreviations provides the information on the total number of carbon atoms and double bonds (CN:DB) of N-linked fatty acyl and sphingoid base of ceramide part.

Gangliosides are present in almost all human tissues, and they are particularly abundant in neural tissues and extraneural organs, such as the spleen, lungs, and in some biological fluids such as blood, and milk [17-22]. Gangliosides play an important role in many physiological processes such as cell signaling, neuronal protection, adhesion, differentiation and dendrite formation [20, 23]. Gangliosides may affect intestinal immunity, tumor progression [19, 24], and they are associated with Alzheimer's disease [25].

1.2 Analysis of lipids

The first and very important step in lipid analysis is extraction of lipids from the sample material. It is very important to gain a stable lipid extraction yield in lipidomic analysis. The standard method of the lipid extraction was introduced by Folch *et al* [26], and later modified by Bligh and Dyer [27]. This method is based on the use of chloroform, methanol, and water as a ternary solvent mixture. More recently modified protocols using (MTBE) [28] and butanol−methanol (BUME) [29] have been developed to improve the extraction process.

The comprehensive lipidomic analysis of sample material such as biological tissues and fluids is a dauting task due to the extreme complexity of the lipidome (amphipathic character of lipids with hydrophobic acyl tails and hydrophilic head groups). For this reason, the separation techniques coupled to MS are widely used for separation and analysis of lipids. Gas chromatography/mass spectrometry (GC/MS) is technique for the analysis of fatty acid methyl esters after the transesterification of lipids, which provides the information on fatty acyls composition. Widely used technique for analysis of lipids is liquid chromatography coupled with MS. LC has relatively wide range of separation modes, which may be tailored for wide range of lipids. Reversed-phase LC can provide complex separation based on the FA length, and also the number and positions of double bonds [19, 21, 30-32]. On the other hand, hydrophilic liquid chromatography (HILIC) separates lipids mainly based on polar head groups. More recently is used ultrahigh-performance supercritical fluid chromatography coupled to MS applied for lipid class separation for nonpolar and polar lipid classes in short analysis times [33]. MS is frequently used analytical technique for qualitative and quantitative analysis due to sensitivity, structural details based on fragmentation of lipids. Three main approaches are often used in lipidomic analysis: (i) direct infusion MS analysis (shotgun) [34],chromatography coupled to MS (LC/MS, GC/MS) [34] and matrix-assisted laser desorption ionization [35].

2. Experimental part

The first goal of this thesis is development and systematic optimization of HILIC/ESI-MS/MS method for the analysis of wide range of gangliosides, describe the fragmentation behavior of individual ganglioside subclasses in MS/MS mode to study elucidate the structure of both oligosaccharide and ceramide parts of ganglioside molecules, and apply the method for the analysis of biological samples.

The next goal of the thesis is the differentiation of renal cell carcinoma tumor and surrounding normal tissues based on the hydrophilic liquid chromatography-electrospray ionization mass spectrometry (HILIC/ESI-MS) quantitation of gangliosides GM3 and other polar lipid classes (sulfohexosylceramides (SulfoHexCer), sulfodihexosylceramides (SulfoHex2Cer), phosphatidylserines (PS), phosphatidylinositols (PI), phosphatidylglycerols (PG), and lysophosphatidylinositols (LPI)) followed by the statistical differentiation of tumor and normal groups, and visualize the most dysregulated lipids.

2.1 Optimization of method and characterization of gangliosides

2.1.1 Chemicals and materials

Methanol, acetonitrile (both HPLC/MS grade), chloroform (HPLC grade, stabilized by 0.5−1% ethanol), ammonium formate, ammonium acetate, formic acid, and acetic acid were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Deionized water was prepared with a Milli-Q Reference Water Purification System (Molsheim, France). The standard of total ganglioside extract from porcine brain was purchased from Avanti Polar

Lipids (Alabaster, AL, U.S.A.). SPE 200 mg of tC18 cartridge (Sep-Pak Vac, 37–55 μm particle size) was purchased from Waters (Waters, Milford, MA, USA). The porcine brain was obtained from the local farm. Samples of plasma, erythrocytes, and kidney were obtained from the Department of Urology, PalackýUniversity, Faculty of Medicine and Dentistry and University Hospital, Olomouc, Czech Republic. The sample of lungs was obtained from the Regional Hospital of Pardubice, Czech Republic. The study was approved by the hospital Ethical Committee, and patients signed documents giving their informed consent.

2.1.2 Sample preparation

Two milligrams of total ganglioside extract from porcine brain was dissolved in 1100 μL of chloroform−methanol−water (600:425:75, v/v/v) mixture. Human blood was collected to heparin-lithium tubes and ultracentrifuged to obtain plasma. Samples of human kidney and human lungs were obtained during surgery, immediately frozen, and stored at −80 °C until the sample processing and the analysis. Human lungs, kidney, erythrocytes, plasma, and porcine brain tissue extracts were obtained by chloroform− methanol−water extraction according to Folch method [26] with minor modifications [36, 37]. Initially, 25 mg of each tissue (human kidney, human lungs, or porcine brain) was cut by scalpel and homogenized in 6 mL of a chloroform−methanol mixture (2:1, v/v) using an ultrasonic bath at 40 °C for 10 min, while human plasma (200 μL) and erythrocytes (200 μL) were homogenized in 3 mL of chloroform−methanol mixture (2:1, v/v) using an ultrasonic bath at 40 °C for 10 min. Then, deionized water (1200 μL for tissues and 600 μL for plasma and erythrocytes) was added, and the mixture was centrifuged at 3000 rpm for 3 min under ambient conditions. The upper aqueous layer containing gangliosides was collected, evaporated by gentle stream of nitrogen to dryness, redissolved in 1 mL of water, and purified by SPE. First, 200 mg of tC18 cartridge was conditioned with 3 mL of methanol followed by 3 mL of water. Then, 1 mL of sample dissolved in water was loaded on the column, washed 3 mL of water, and finally eluted by 3 mL of methanol. The eluate was collected, then evaporated by a gentle stream of nitrogen to dryness and redissolved in 500 μL of methanol−water− chloroform (300:150:50, v/v/v) mixture for analysis.

2.1.3 LC/MS conditions

All LC experiments were performed on a liquid chromatograph Agilent 1290 Infinity series (Agilent Technologies, Waldbronn, Germany). The final method for the analysis of individual lipid subclasses used the following conditions: Ascentis Si column (150 × 2.1, 3 μm, Sigma-Aldrich), flow rate 0.3 mL/min, injection volume 1 μL, column temperature 40 °C, and mobile-phase gradient as follows□0 min: 87.7% A + 12.3% B; 15 min: 77.9% A + 22.1% B, where phase A was acetonitrile with acetic acid, and phase B was 10 mM aqueous ammonium acetate with pH 6.1 adjusted by acetic acid.

The setting of hybrid quadrupole time-of-flight mass spectrometer (micrOTOF-Q, Bruker Daltonics, Bremen, Germany) in ESI mode was used: capillary voltage 2.5 kV, nebulizing gas pressure 1.2 bar, drying gas flow rate 9.3 L/min, and drying gas temperature 210 °C, mass range 50 – 300 *m/z*. The hybrid quadrupole traveling wave ion mobility time

of flight mass spectrometer Synapt G2Si (Waters) in the resolution mode was used for the identification with the following conditions: negative-ion ESI, mass range m/z 50− 2000, capillary voltage 2.2 kV, sampling cone 20 V, source offset 90 V, source temperature 150 °C, drying temperature 500 °C, cone gas flow 0.8 L/min, drying gas flow 17 L/min, and nebulizer gas flow 4 bar. Leucine enkephaline was used as the lock mass for all experiments. MS/MS experiments were performed on the transfer cell with the collision energy ramp from 20 to 70 eV.

2.1.4 Results and discussion

Ascentis Si column has been used for the separation of gangliosides. The standard of total ganglioside extract from porcine brain was used for the optimization of these major ganglioside subclasses: GM1a, Fuc-GM1a, GD1a, GD1b, and GT1b. Ammonium acetate and ammonium formate have been used for LC/MS optimization from 0 to 30 mmol/L. The

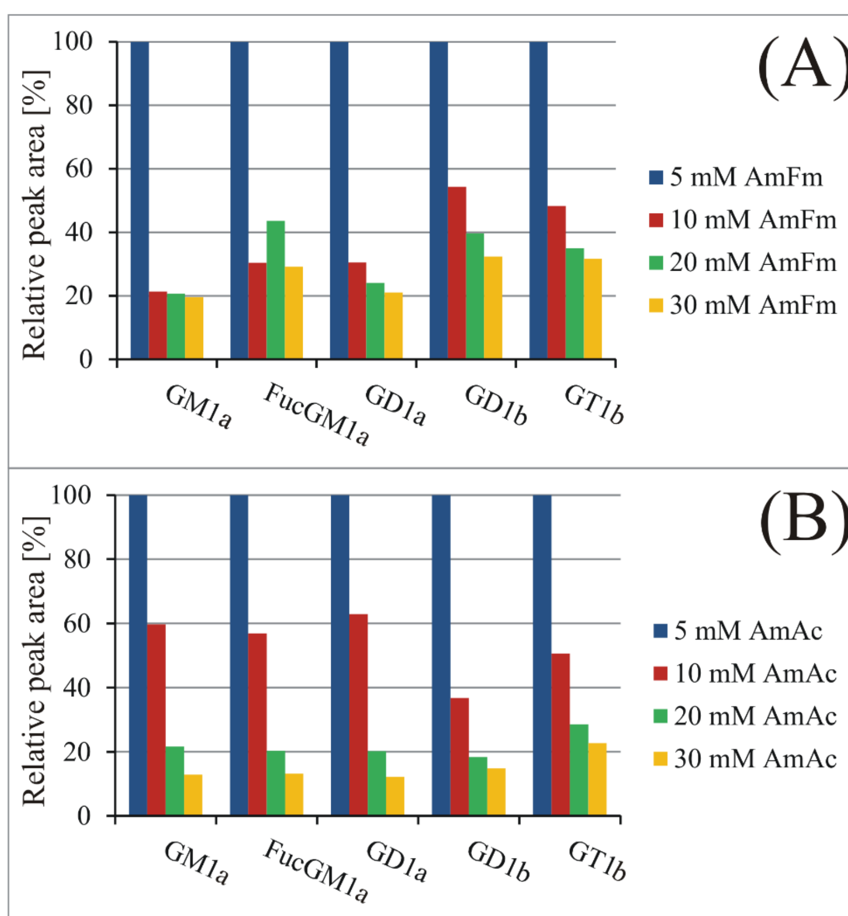

Figure 2: Comparison of relative peak areas vs molar concentrations of (A) ammonium formate (AmFm) and (B) ammonium acetate (AmAc) for major individual gangliosides GM1a 36:1 (RIC of [M− H]− at m/z 1544.9), Fuc−GM1a 36:1 (RIC of [M−H]− at m/z 1690.9), GD1a 36:1 (RIC of [M−H]2− at m/z 917.5), GD1b 36:1 (RIC of [M−H]2− at m/z 917.5), and GT1b 38:1 (RIC of [M−H]2− at m/z 1077.0).

highest signal for ammonium acetate and ammonium formate was observed at 5 mM (Figure 2), but the best compromise between the sensitivity and peak shape was 10 mM. Mobile phases without additives cannot be used for the separation due to the unacceptable peak tailing and no chromatographic resolution of isomeric GD1a and GD1b.

The next step in optimization of HILIC/ESI-MS method was testing of pH mobile phase. The pH range from 3.0 to 5.8 was tested for 10 mM of ammonium formate using the addition of formic acid and from 3.0 to 6.5 for 10 mM of ammonium acetate using the addition of acetic acid. The direct comparison of optimum conditions for both tested buffers in Figure 3C shows that the highest signal was obtained for ammonium acetate at pH 6.1. The peak width and tailing factors were also considered, where the worst results are obtained for most acidic mobile phase (pH 3) without any resolution of GD1a and GD1b. Measurements for all other pH values provided comparable results. Therefore, the best compromise among the sensitivity, tailing factors, and the chromatographic resolution is 10 mM of ammonium acetate, because it also provides a partial separation of Fuc−GM1a

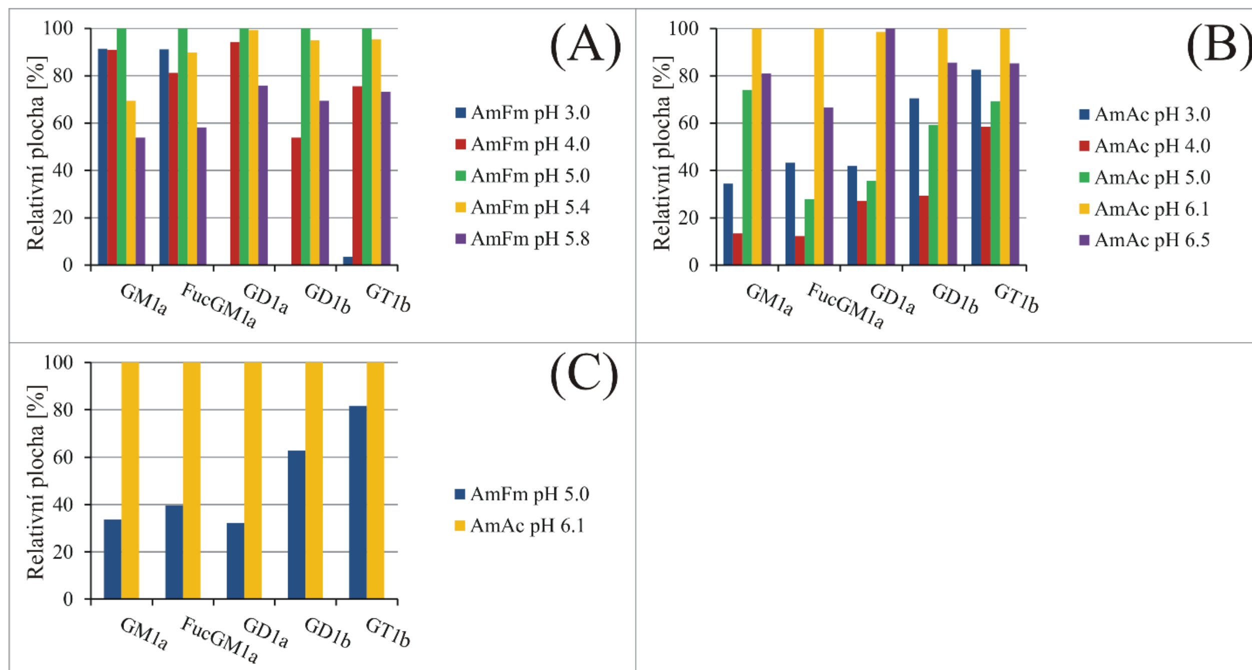

Figure 3: Comparison of relative peak areas *vs.* pH for (A) 5 mmol/L ammonium formate (AmFm) and (B) 10 mmol/L ammonium acetate (AmAc) and comparison of (C) optimal pH of ammonium formate and ammonium acetate for major individual gangliosides: GM1 36:1 (RIC of $[M-H]^-$ at *m/z* 1544.9), FucGM1 36:1 (RIC of $[M-H]^-$ at *m/z* 1690.9), GD1a 36:1 (RIC of $[M-H]^{2-}$ at *m/z* 917.5), GD1b 36:1 (RIC of $[M-H]^{2-}$ at *m/z* 917.5), and GT1b 38:1 (RIC of $[M-H]^{2-}$ at *m/z* 1077.0). GD1a and GD1b are not separated at pH 3.0 of ammonium formate buffer.

and GD1a unlike to 5 mM of ammonium acetate, but at cost of reduced sensitivity and longer analysis time.

The final method was used for the analysis of porcine brain extract (Figure 4). The 19 ganglioside subclasses and 5 other polar lipid classes were identified in porcine brain extract (Figure 5). The identification was provided based on the retention time, and accurate mass *m/z* of precursor and fragment ions. The full scan negative-ion ESI mass spectra were obtained for all peaks corresponding to individual subclasses of gangliosides, and the elemental composition were determined on the basis of accurate m/z measurements with the average mass accuracy of 3.1 ppm. Then, MS/MS spectra of $[M-H]^{-}$, $[M-2H]^{2-}$, or $[M-3H]^{3-}$ ions were recorded and interpreted. The fragmentation behavior of gangliosides provided an excellent tool for sequencing of oligosaccharide part. The fragmentation was predictable and therefore provided unambiguous information on the sequence including the type of branching, i.e., the differentiation of *a, b,* and *c* series (Figure 5).

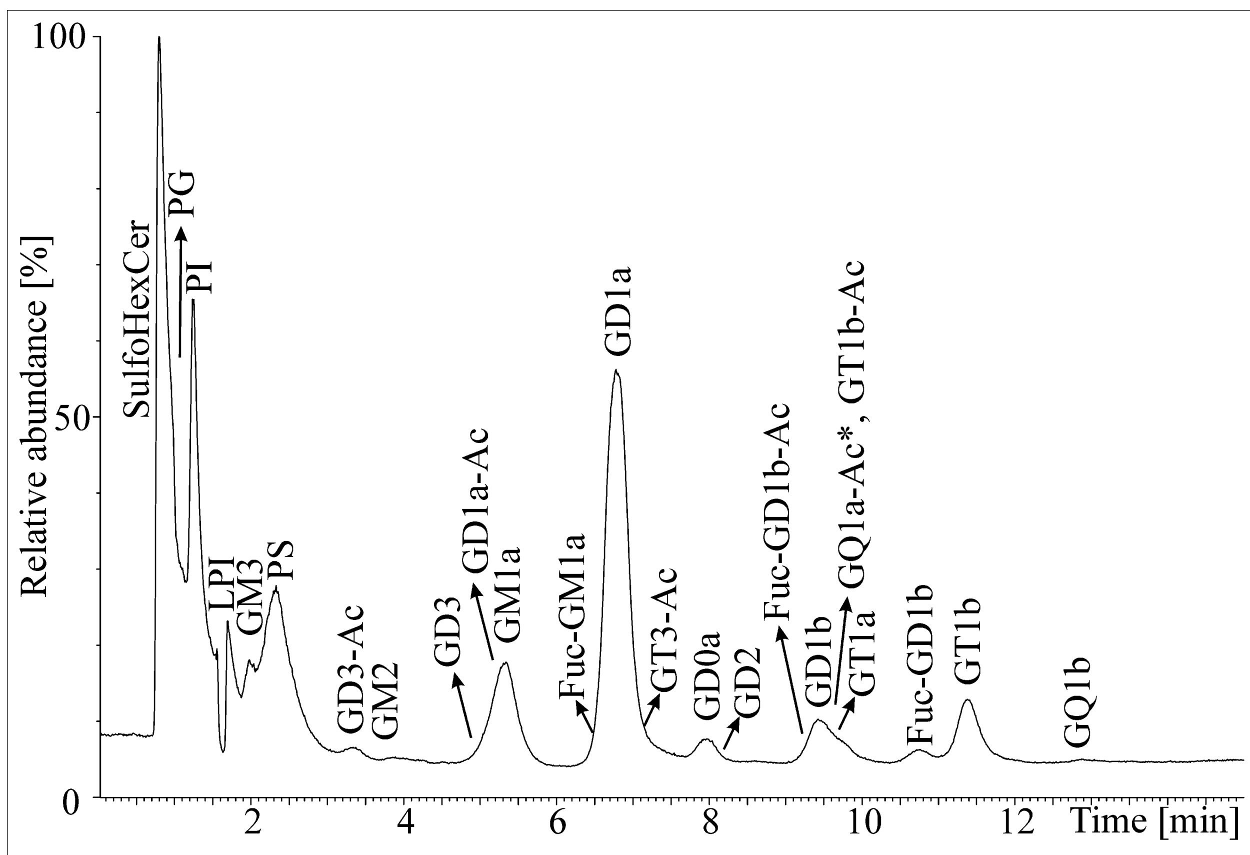

Figure 4: Negative-ion HILIC/ESI-MS total ion current chromatogram of porcine brain extract. Conditions: Ascentis Si column (150 × 2.1, 3 μm, Sigma-Aldrich), flow rate 0.3 mL/min, injection volume 1 μL, column temperature 40 °C, and mobile-phase gradient-0 min: 87.7% A + 12.3% B; 15 min: 77.9% A + 22.1% B, where phase A was acetonitrile with acetic acid, and phase B was 10 mM aqueous ammonium acetate with pH 6.1 adjusted by acetic acid.

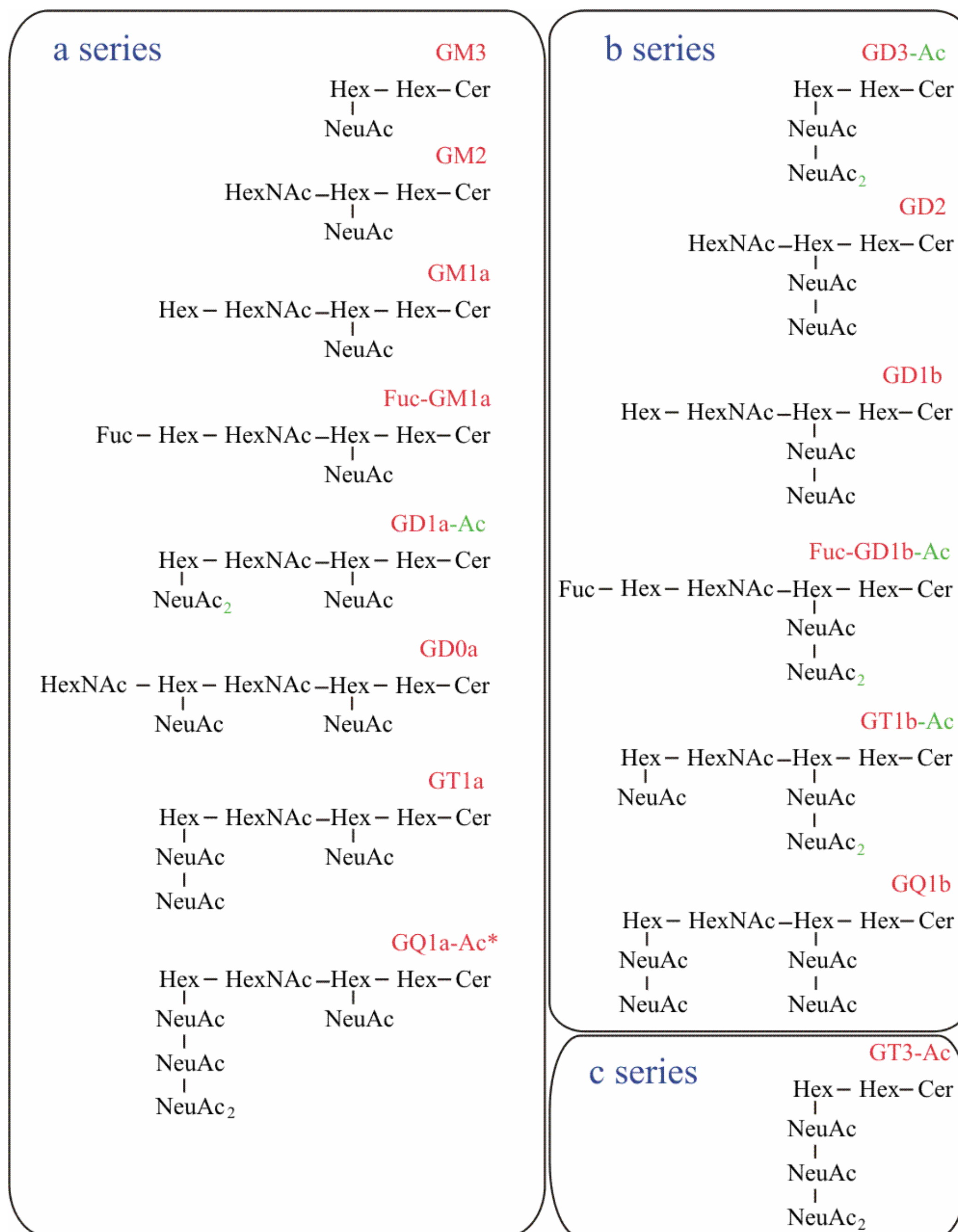

Figure 5: Structures of gangliosides identified in this work. Annotation:[16] the first letter G means ganglioside, the second letter indicates the number of sialic acids (M = mono, D = di, T = tri, and Q = tetra), then the number of neutral saccharide units is calculated as 5−n, the series a is −Hex(NeuAc)−Hex−Cer, the series b is −Hex(NeuAc−NeuAc)−Hex−Cer, and the series c is −Hex- (NeuAc−NeuAc−NeuAc)−Hex−Cer. The asterisk (GQ1a−Ac*) means that the structure is not confirmed by MS/MS spectra.

The final method was applied for measurements of the porcine brain extract and also human kidney, lungs, plasma, erythrocytes extracts. The gangliosides that are identified with high confidence were reported considering the following criteria: (1) accurate *m/z* values to determine the elemental composition (99% of measurements <10 ppm, 87% of measurements <5 ppm, and the average mass accuracy 3.1 ppm), (2) the interpretation of fragmentation behavior to sequence the oligosaccharide part of the molecule based on accurate *m/z* values, and (3) retention time. The table clearly showed that the brain tissue contains by far more gangliosides than other studied biological samples. Only GM3 were detected for all studied sample types with only few additional gangliosides in case of kidney (3 GD3−Ac, 3 GM2, 9 GD3, and 4 GD1a), lungs (2 GM2, 10 GD3, and 2 GD1a), and erythrocytes (4 GM1a). In total, 25 classes of lipids and 272 lipids species were identified in human kidneys, lungs, plasma, erythrocytes and porcine brain (Table 1). The largest number of identified lipids was in porcine brain, where 38 sulfatides, 42 phospholipids and 109 gangliosides were identified from 189 lipids. In human kidney were identified 51 sulfatides, 39 phospholipids and 32 gangliosides. 3 sulfatides, 30 phospholipids and 34 gangliosides were identified in human lungs. A lower number of lipids than in tissues has been identified in human fluids. In human plasma were identified 2 sulfatides, 13 phospholipids and 15 gangliosides belonging to the GM3 class. In human erythrocytes were identified 8 sulfatides, 19 phospholipids and 9 gangliosides.

Table 1: Overview of lipids identified in porcine brain, human kidney, lungs, plasma, and erythrocyte extracts using HILIC/ESI-MS/MS method.

Lipid class	Retention time window [min]	Sample type					Total
		Porcine brain	Human kidney	Human lungs	Human plasma	Human erythrocytes	
SulfoHexCer	0.6 - 1.7	38	25	3	2	4	43
$SulfoHex_2Cer$	0.6 - 1.7	-	25	-	-	4	25
PG	1.0 - 1.5	7	4	5	-	-	9
PI	1.0 - 1.5	10	20	13	8	9	20
LPI	1.7 - 2.4	8	8	4	5	-	10
PS	2.0 - 2.9	17	7	8	-	10	20
GM3	1.9 - 2.5	2	16	15	15	4	22
GD3-Ac	2.8 - 3.8	13	-	2	-	-	14
GM2	3.3 - 4.1	3	3	2	-	-	6
GD3	4.5 - 5.7	9	9	13	-	-	17
GD1a-Ac	4.8 - 5.5	3	-	-	-	-	3
GM1a	4.9 - 5.8	10	-	-	-	5	11
Fuc-GM1a	6.2 - 7.2	7	-	-	-	-	7
GD1a	6.3 - 7.1	7	4	2	-	-	10
GT3-Ac	6.3 - 7.3	6	-	-	-	-	6
GD0a	7.3 - 8.3	7	-	-	-	-	7
GD2	7.5 - 8.4	2	-	-	-	-	2
Fuc-GD1b-Ac	8.3 - 9.0	3	-	-	-	-	3
GD1b	8.8 - 9.6	8	-	-	-	-	8
GT1b-Ac	9.2 - 9.9	4	-	-	-	-	4
GQ1-Ac	9.2 - 9.9	2	-	-	-	-	2
GT1a	9.3 - 9.9	4	-	-	-	-	4
Fuc-GD1b	10.3 - 11.3	5	-	-	-	-	5
GT1b	10.8 – 12.0	12	-	-	-	-	12
GQ1b	12.4 - 13.5	2	-	-	-	-	2
Total		189	122	67	30	36	272

2.2 Differentiation of RCC tumor and surrounding normal tissues

The incidence of renal cell carcinoma (RCC) has been increasing in recent decades [38, 39]. RCC currently ranks among the ten most common cancers in industrialized countries, representing around 2% of all malignant neoplasms [38-40]. RCC originates from the renal epithelium and accounts for more than 90% of kidney tumors [41]. More than ten histological and molecular RCC subtypes have been described so far, of which clear cell RCC (ccRCC) represents an overwhelming majority of cases and accounts for most RCC-related deaths [41] with a prevalence of 75% of all primary kidney cancers [42]. Determination of the most significant changes in lipid composition between RCC tumor and surrounding normal tissues was based on HILIC/ESI-MS analysis of samples obtained from 20 patients with ccRCC.

2.2.1 Chemicals and materials

Acetonitrile, methanol (both HPLC/MS grade), chloroform (HPLC grade, stabilized by 0.5–1% ethanol), ammonium acetate, acetic acid, lactosyl sphingosine, cytidine-5′-monophospho-N-acetylneuraminic acid sodium salt, and α2,3-sialyltransferase from Pasteurella multocida were purchased from Sigma-Aldrich (St. Louis, MO, USA). SPE 200 mg of tC18 cartridge (Sep-Pak Vac, 37–55 µm particle size) was purchased from Waters (Waters, Milford, MA, USA). Deionized water was prepared with a Milli-Q Reference Water Purification System (Molsheim, France). Standards of SulfoHexCer d18:1/12:0 and PS 14:0/14:0 used as internal standards (IS) for the method validation and quantitation were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Synthesis of internal standard GM3 d18:1/12:0 is describe in publication of *HILIC/ESI-MS determination of gangliosides and other polar lipid classes in renal cell carcinoma and surrounding normal tissues* [5]. Stock solutions of individual IS at the concentration of 1 mg/mL for GM3, SulfoHexCer, and PS were prepared in a methanol-water-chloroform mixture (6:3:1, v/v/v). 20 male patients with ccRCC were analyzed in the present study. RCC tumor tissue and surrounding normal tissue (classification confirmed by histology) were analyzed for each ccRCC patient. The mean (± standard deviation) age of patients was 61.7 ± 11.5 years, and the mean body mass index (BMI) was 28.9 ± 7.5 kg/m^2. The study was approved by the hospital Ethical Committee, and patients signed informed consent.

2.2.2 Sample preparation

Human tissues od RCC tumor and surrounding normal tissues were extracted according to Folch [26] method with minor modifications [43, 44]. Initially, 25 mg of kidney tissue spiked with internal standards was homogenized in 6 mL of chloroform/methanol mixture (2:1, v/v) using an ultrasonic bath at 40 °C for 10 min. Then, 1200 µL of deionized water was added, and the mixture was centrifuged at 3000 rpm for 3 min. The upper aqueous layer containing polar compounds was collected, evaporated by gentle stream of nitrogen, redissolved in 1 mL of water, and finally purified by SPE. SPE was conditioned with 3 mL of methanol followed by 3 mL of water. Then, 1 mL of sample

dissolved in water was loaded on the cartridge, washed with 3 mL of water. Then, 1 mL of sample dissolved in water was loaded on the column, washed 3 mL of water, and finally eluted by 3 mL of methanol. The eluate was collected, then evaporated by a gentle stream of nitrogen to dryness and redissolved in 500 µL of methanol−water−chloroform (300:150:50, v/v/v) mixture for analysis.

2.2.3 LC/MS conditions

HILIC/ESI-MS experiments were performed on a liquid chromatograph Agilent 1290 Infinity series (Agilent Technologies, Waldbronn, Germany). The final method for the analysis of lipids used the following conditions: Ascentis Si column (150 × 2.1, 3 µm, Sigma-Aldrich), the flow rate 0.3 mL/min, the column temperature 40 °C, and the mobile phase gradient: 0 min—95% A + 5% B, 10 min—78% A + 22% B, where phase A was acetonitrile with acetic acid, and phase B was 10 mM aqueous ammonium acetate with acetic acid (0.5 µL of acetic acid was added to 100 mL of both A and B phases). The analytical experiments were performed on Xevo G2- XS QTOF mass spectrometer (Waters, Milford, MA, USA). Data were acquired in the sensitivity mode using negative-ion ESI using the typical resolving power 22,000 (full width at half-maximum). The acquisition range was *m/z* 50–2000. The capillary voltage was 1 kV, the source temperature 150 °C, the cone gas flow 50 L/h, the desolvation gas flow 1000 L/h, and the scan time 0.5 s. MassLynx Software was used for the data acquisition.

Acquired data were processed using MarkerLynx XS software. Individual ESI full scans corresponding to the lipid class peak were combined together with the peak separation 50 mDa and markers extracted with the intensity threshold 3000. Then, the data in *m/z* vs. intensity format were transferred into the LipidQuant software. The statistical evaluation was performed by MDA methods, such as PCA and OPLS-DA in the SIMCA software (version 13.0, Umetrics, Umeå, Sweden).

2.2.4 Results and discussion

Ascentis Si column was selected for the determination of ganglioside due to the good chromatographic performance in our previous study. The gradient conditions were optimized to achieve short chromatographic time for GM3 and other polar lipid classes. The optimized final method was validated for the quantitative analysis of GM3, SulfoHexCer, and PS in kidney tumor and surrounding normal tissues. Internal standards were used with shorter fatty acyls, such as 14:0/14:0 for PS and the combination of 18:1/12:0 in case of SulfoHexCer and GM3 because these combinations of fatty acyls were not detected in the pooled sample of kidney tissues. Mentioned IS were used for the method validation. Extract from pooled kidney with mixture of IS was used for the determination of system suitability, linearity range, calibration slope, LOD, and LOQ.

The lipids were identified based on retention time and accurate *m/z* value of $[M-H]^-$ ions measured by QTof mass analyzer (Figure 6). The quantitative analysis of SulfoHexCer, SulfoHex2Cer, GM3, and PS classes was based on the peak intensity normalized to coeluting lipid class IS. Other glycerophospholipid classes (PI, LPI, and PG) were quantified using IS of PS 14:0/14:0, which was eluted at different retention times,

therefore, the quantitative data have to be reported as semi-quantitative, although it still provides valuable information about the dysregulation of particular lipids.

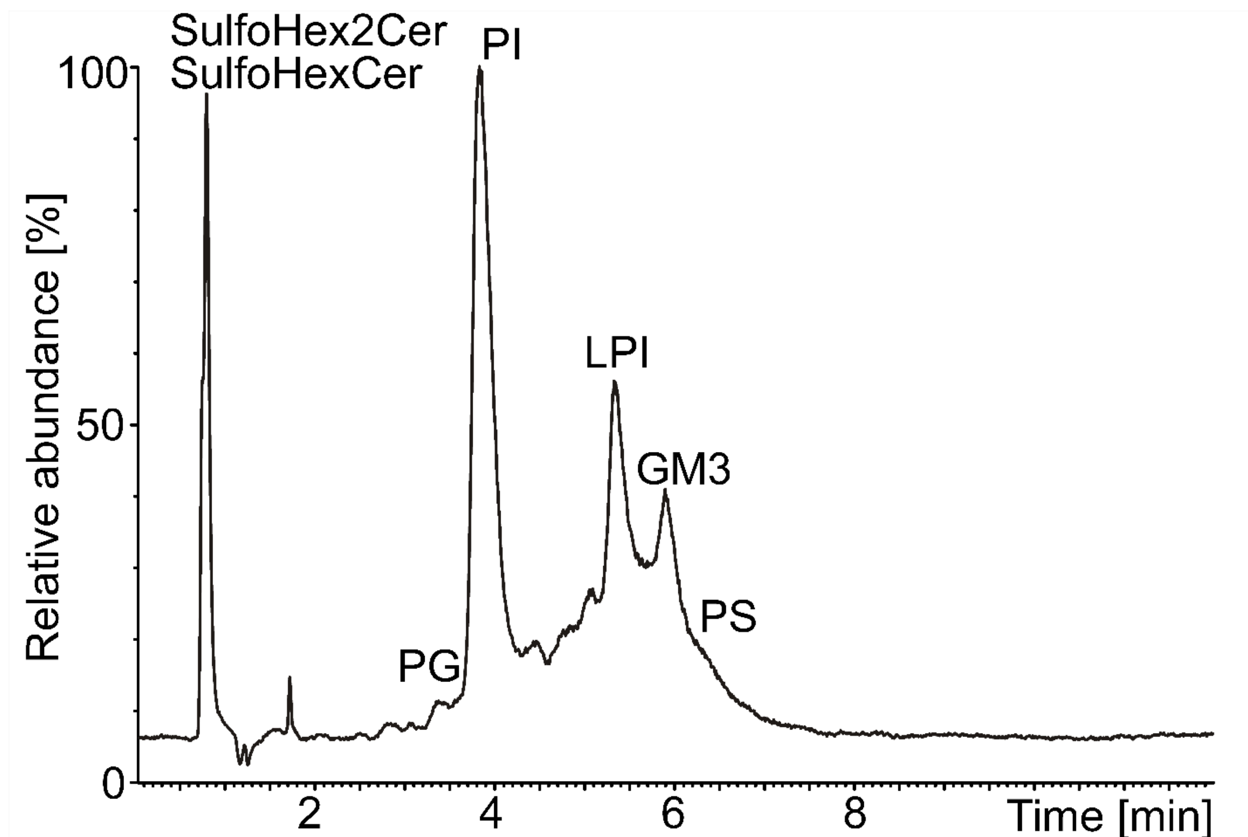

Figure 6: Typical example of negative-ion HILIC/ESI-MS chromatogram of kidney tissue extract with the annotation of individual quantified lipid classes, including sulfohexosylceramides (SulfoHexCer), sulfodihexosylceramides (SulfoHex2Cer), phosphatidylglycerols (PG), phosphatidylinositols (PI), lysophosphatidylinositols (LPI), ganglioside (GM3), and phosphatidylserines (PS).

The data were processed by unsupervised PCA method (Fig. 7a), which confirms the normal distribution of data set and indicates relatively good clustering of tumor samples (red points) and surrounding normal tissue samples (blue points). In the next step, the data were processed by supervised OPLS-DA, which showed excellent clustering of tumor samples (red points) and surrounding normal tissue samples (blue points). S-plot (Fig. 7c) created from supervised OPLS-DA method provides the information on the most upregulated (upper right part) and downregulated (bottom left part) lipids in the tumor samples. The central part of S-plot showed lipids with a low statistical significance; therefore, they were not annotated. The most upregulated lipids in the tumor were PI 40:5, PI 40:4, GM3 41:1, GM3 36:2, GM3 41:2, GM3 42:3, GM3 34:1, and GM3 42:2, while the most downregulated ones were SulfoHexCer 41:1 (OH), PS 36:4, SulfoHexCer 38:1 (OH), PI 34:0, LPI 16:0, SulfoHexCer 40:1, SulfoHexCer 42:0 (2*OH), and SulfoHexCer 42:1 (OH).

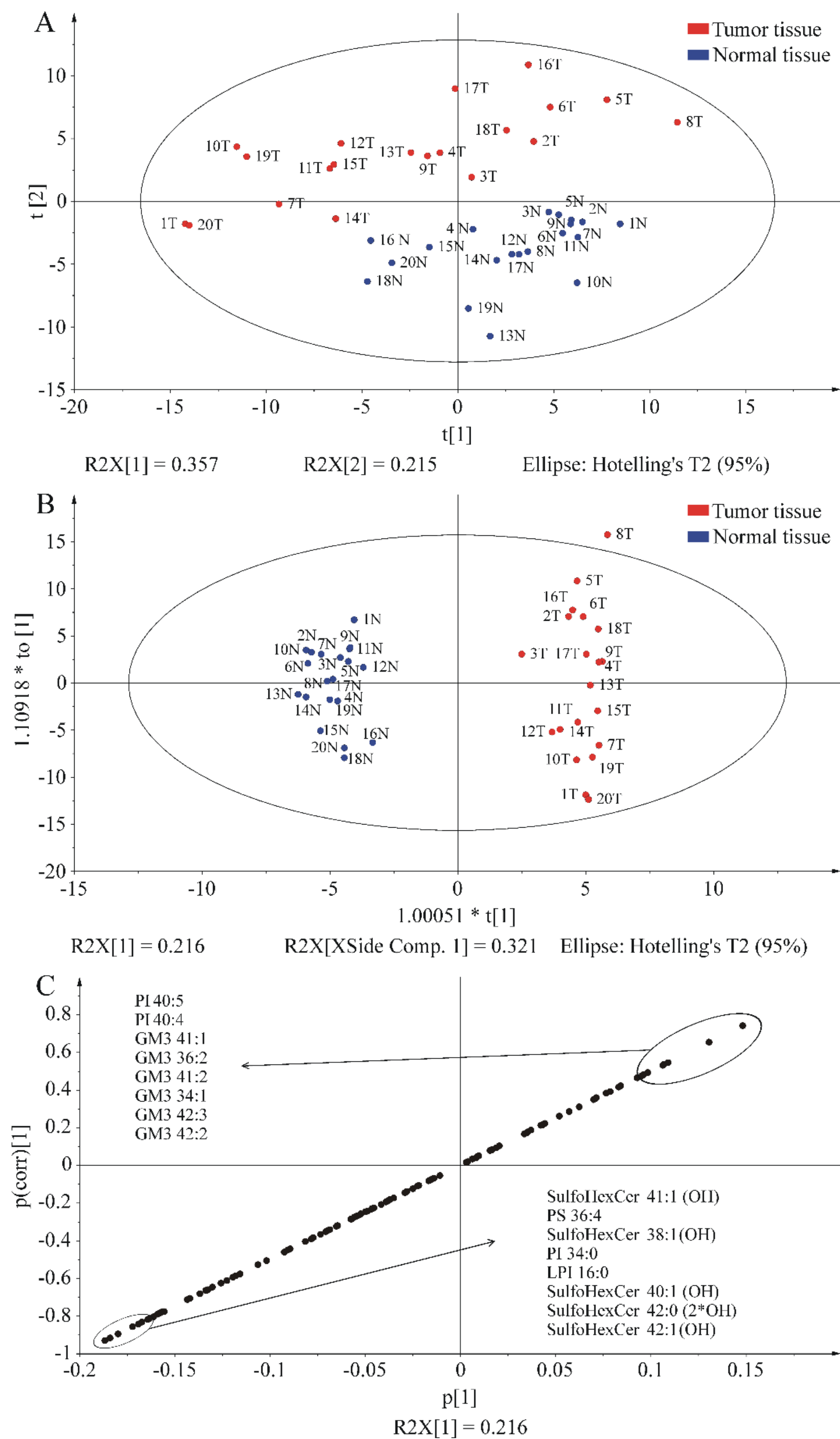

Figure 7: Multivariate data analysis of lipidomic data for RCC tumor (red) and surrounding normal tissues (blue) measured for 20 patients. a Unsupervised PCA score plot. b Supervised OPLS score plot. c S-plot with the annotation of most up- and downregulated lipids.

3. Conclusion

This doctoral thesis deals with the analysis of lipids in biological materials. The work was divided into two main sections – develop method for analysis of the gangliosides, and identification of gangliosides, determination of gangliosides and other polar lipid classes in renal cell carcinoma and surrounding normal tissues.

In the first part, HILIC/ESI-MS/MS method enabled identification of the highest number of gangliosides in human kidney, lungs, plasma, erythrocytes, and porcine brain. The method enabled identified 145 ganglioside molecular species from 19 subclasses with additional identification of other 6 polar lipid subclasses (SulfoHexCer, SulfoHex2Cer, PG, PI, LPI, and PS), which are known to be difficult for established MS-based lipidomic platforms. The gangliosides that are identified with high confidence was identified based on accurate *m/z* values of precursors and product ions, the agreement of fragmentation behavior in MS/MS with structure assignment, the correlation with the predicted retention behavior of ganglioside subclasses, and also ganglioside species inside the individual subclass.

The HILIC/ESI-MS method was optimized and fully validated according FDA and EMA recommendation. The quantitative method was used for analysis of selected gangliosides, sulfatides and polar phospholipids. The method was used for determination of 115 lipid species from seven classes (GM3, SulfoHexCer, SulfoHex2Cer, PG, PI, LPI, and PS) in extract of 40 samples of RCC tumor tissues and surrounding normal tissues of RCC patients after the surgery. MDA showed a clear distinction of tumor and normal tissues using supervised OPLS-DA approach; however, the full resolution was observed already in non-supervised PCA graph, which illustrated very significant differences of both groups based on the lipidomic analysis. Phospholipid classes PI, LPI, and PG are only semi-quantified using PS 14:0/14:0 due to the lack of their class IS, but regardless this limitation, the data show the upregulation of highly unsaturated PI 40:4 and 40:5 and the downregulation of saturated PI 32:0, PI 34:0, and LPI 16:0.

4. List of References

[1] M. Holčapek, G. Liebisch, K. Ekroos, Lipidomic analysis, in, ACS Publications, 2018.
[2] E. Fahy, S. Subramaniam, H.A. Brown, C.K. Glass, A.H. Merrill Jr, R.C. Murphy, C.R. Raetz, D.W. Russell, Y. Seyama, W. Shaw, A comprehensive classification system for lipids, European Journal of Lipid Science and Technology, 107 (2005) 337-364.
[3] S.-I. Hakomori, Bifunctional role of glycosphingolipids. Modulators for transmembrane signaling and mediators for cellular interactions, Journal of Biological Chemistry, 265 (1990) 18713-18716.
[4] M. Aureli, L. Mauri, M.G. Ciampa, A. Prinetti, G. Toffano, C. Secchieri, S. Sonnino, GM1 ganglioside: past studies and future potential, Molecular neurobiology, 53 (2016) 1824-1842.
[5] R. Hájek, M. Lísa, M. Khalikova, R. Jirásko, E. Cífková, V. Študent, D. Vrána, L. Opálka, K. Vávrová, M. Matzenauer, HILIC/ESI-MS determination of gangliosides and other polar lipid classes in renal cell carcinoma and surrounding normal tissues, Analytical and bioanalytical chemistry, 410 (2018) 6585-6594.
[6] S. Sonnino, L. Mauri, M.G. Ciampa, A. Prinetti, Gangliosides as regulators of cell signaling: ganglioside-protein interactions or ganglioside-driven membrane organization?, Journal of neurochemistry, 124 (2013) 432-435.
[7] T. Takahashi, T. Suzuki, Role of sulfatide in normal and pathological cells and tissues, Journal of lipid research, 53 (2012) 1437-1450.
[8] K. Yang, X. Han, Lipidomics: techniques, applications, and outcomes related to biomedical sciences, Trends in biochemical sciences, 41 (2016) 954-969.
[9] E. Fahy, S. Subramaniam, H.A. Brown, C.K. Glass, A.H. Merrill, R.C. Murphy, C.R.H. Raetz, D.W. Russell, Y. Seyama, W. Shaw, T. Shimizu, F. Spener, G. van Meer, M.S. VanNieuwenhze, S.H. White, J.L. Witztum, E.A. Dennis, A comprehensive classification system for lipids, Journal of Lipid Research, 46 (2005) 839-862.
[10] X. Chen, A. Varki, Advances in the biology and chemistry of sialic acids, ACS chemical biology, 5 (2010) 163-176.
[11] M. Scherer, G. Schmitz, G. Liebisch, High-throughput analysis of sphingosine 1-phosphate, sphinganine 1-phosphate, and lysophosphatidic acid in plasma samples by liquid chromatography–tandem mass spectrometry, Clinical chemistry, 55 (2009) 1218-1222.
[12] R.L. Schnaar, R. Gerardy-Schahn, H. Hildebrandt, Sialic acids in the brain: gangliosides and polysialic acid in nervous system development, stability, disease, and regeneration, Physiological Reviews, 94 (2014) 461-518.
[13] R. Schauer, Sialic acids: fascinating sugars in higher animals and man, Zoology, 107 (2004) 49-64.
[14] Y.N. Malykh, R. Schauer, L. Shaw, N-Glycolylneuraminic acid in human tumours, Biochimie, 83 (2001) 623-634.
[15] L. Svennerholm, CHROMATOGRAPHlC SEPARATION OF HUMAN BRAIN GANGLIOSIDES, Journal of neurochemistry, 10 (1963) 613-623.

[16] M. Chester, IUPAC-IUB Joint Commission on Biochemical Nomenclature (JCBN). Nomenclature of glycolipids--recommendations 1997, European journal of biochemistry, 257 (1998) 293.
[17] R. Lacomba, J. Salcedo, A. Alegría, M.J. Lagarda, R. Barberá, E. Matencio, Determination of sialic acid and gangliosides in biological samples and dairy products: a review, Journal of pharmaceutical and biomedical analysis, 51 (2010) 346-357.
[18] T. Valdes-Gonzalez, N. Goto-Inoue, W. Hirano, H. Ishiyama, T. Hayasaka, M. Setou, T. Taki, New approach for glyco-and lipidomics–Molecular scanning of human brain gangliosides by TLC-Blot and MALDI-QIT-TOF MS, Journal of neurochemistry, 116 (2011) 678-683.
[19] F. Giuffrida, I.M. Elmelegy, S.K. Thakkar, C. Marmet, F. Destaillats, Longitudinal evolution of the concentration of gangliosides GM3 and GD3 in human milk, Lipids, 49 (2014) 997-1004.
[20] T. Kaneko, Y. Tsubakihara, H. Fushimi, S. Yamaguchi, Y. Takabatake, H. Rakugi, H. Kawakami, Y. Isaka, Histochemical and immunoelectron microscopic analysis of ganglioside GM3 in human kidney, Clinical and experimental nephrology, 19 (2015) 403-410.
[21] M. Fuller, S. Duplock, L.K. Hein, B.A. Rigat, D.J. Mahuran, Liquid chromatography/electrospray ionisation–tandem mass spectrometry quantification of GM2 gangliosides in human peripheral cells and plasma, Analytical biochemistry, 458 (2014) 20-26.
[22] L. Ma, A.K. MacGibbon, H.J.B.J. Mohamed, S. Loy, A. Rowan, P. McJarrow, B.Y. Fong, Determination of ganglioside concentrations in breast milk and serum from Malaysian mothers using a high performance liquid chromatography-mass spectrometry-multiple reaction monitoring method, International Dairy Journal, 49 (2015) 62-71.
[23] A.D. Garcia, J.L. Chavez, Y. Mechref, Rapid and sensitive LC-ESI-MS of gangliosides, Journal of Chromatography B, 947 (2014) 1-7.
[24] S. Fougeray, J. Fleurence, S. Faraj, M. Bahri, D. Cochonneau, M. Terme, M.-D. Leclair, E. Thébaud, F. Paris, S. Birklé, O-acetylated gangliosides: Structure, biosynthesis, immunogenicity, functions and their potential for cancer immunotherapy, Journal of Cancer Research and Therapeutics, 4 (2016) 21-30.
[25] L. Svennerholm, G. Bråne, I. Karlsson, A. Lekman, I. Ramström, C. Wikkelsö, Alzheimer disease–effect of continuous intracerebroventricular treatment with GM1 ganglioside and a systematic activation programme, Dementia and geriatric cognitive disorders, 14 (2002) 128-136.
[26] J. Folch, M. Lees, G. Sloane Stanley, A simple method for the isolation and purification of total lipids from animal tissues, J biol Chem, 226 (1957) 497-509.
[27] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, Canadian journal of biochemistry and physiology, 37 (1959) 911-917.
[28] V. Matyash, G. Liebisch, T.V. Kurzchalia, A. Shevchenko, D. Schwudke, Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics, Journal of lipid research, 49 (2008) 1137-1146.

[29] 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, Journal of lipid research, 53 (2012) 1690-1700.
[30] K. Ikeda, T. Shimizu, R. Taguchi, Targeted analysis of ganglioside and sulfatide molecular species by LC/ESI-MS/MS with theoretically expanded multiple reaction monitoring, Journal of lipid research, 49 (2008) 2678-2689.
[31] B. Fong, C. Norris, E. Lowe, P. McJarrow, Liquid Chromatography–High-Resolution Mass Spectrometry for Quantitative Analysis of Gangliosides, Lipids, 44 (2009) 867-874.
[32] L.K. Sørensen, A liquid chromatography/tandem mass spectrometric approach for the determination of gangliosides GD3 and GM3 in bovine milk and infant formulae, Rapid Communications in Mass Spectrometry: An International Journal Devoted to the Rapid Dissemination of Up-to-the-Minute Research in Mass Spectrometry, 20 (2006) 3625-3633.
[33] M. Lísa, M. Holčapek, High-throughput and comprehensive lipidomic analysis using ultrahigh-performance supercritical fluid chromatography–mass spectrometry, Analytical Chemistry, 87 (2015) 7187-7195.
[34] M. Lísa, E. Cífková, M. Khalikova, M. Ovčačíková, M. Holčapek, Lipidomic analysis of biological samples: comparison of liquid chromatography, supercritical fluid chromatography and direct infusion mass spectrometry methods, Journal of Chromatography A, 1525 (2017) 96-108.
[35] 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, Journal of The American Society for Mass Spectrometry, 28 (2017) 1562-1574.
[36] E. Cífková, M. Holčapek, M. Lísa, M.n. Ovčačíková, A.n. Lyčka, F.d.r. Lynen, P. Sandra, Nontargeted quantitation of lipid classes using hydrophilic interaction liquid chromatography–electrospray ionization mass spectrometry with single internal standard and response factor approach, Analytical Chemistry, 84 (2012) 10064-10070.
[37] R. Hájek, R. Jirásko, M. Lísa, E. Cífková, M. Holčapek, Hydrophilic Interaction Liquid Chromatography–Mass Spectrometry Characterization of Gangliosides in Biological Samples, Analytical Chemistry, 89 (2017) 12425-12432.
[38] J.E. Lee, D. Spiegelman, D.J. Hunter, D. Albanes, L. Bernstein, P.A. Van Den Brandt, J.E. Buring, E. Cho, D.R. English, J.L. Freudenheim, Fat, protein, and meat consumption and renal cell cancer risk: a pooled analysis of 13 prospective studies, JNCI: Journal of the National Cancer Institute, 100 (2008) 1695-1706.
[39] P. Li, A. Znaor, I. Holcatova, E. Fabianova, D. Mates, M.B. Wozniak, J. Ferlay, G. Scelo, Regional geographic variations in kidney cancer incidence rates in European countries, European urology, 67 (2015) 1134-1141.
[40] R. Bellocco, E. Pasquali, M. Rota, V. Bagnardi, I. Tramacere, L. Scotti, C. Pelucchi, P. Boffetta, G. Corrao, C. La Vecchia, Alcohol drinking and risk of renal cell carcinoma: results of a meta-analysis, Annals of oncology, 23 (2012) 2235-2244.
[41] J.J. Hsieh, M.P. Purdue, S. Signoretti, C. Swanton, L. Albiges, M. Schmidinger, D.Y. Heng, J. Larkin, V. Ficarra, Renal cell carcinoma, Nature reviews Disease primers, 3 (2017) 17009.

[42] N. Petejova, A. Martinek, Renal cell carcinoma: Review of etiology, pathophysiology and risk factors, Biomedical Papers of the Medical Faculty of Palacky University in Olomouc, 160 (2016).
[43] E. Cífková, M. Holčapek, M. Lísa, D. Vrána, B. Melichar, V. Študent, Lipidomic differentiation between human kidney tumors and surrounding normal tissues using HILIC-HPLC/ESI–MS and multivariate data analysis, Journal of Chromatography B, 1000 (2015) 14-21.
[44] M. Ovčačíková, M. Lísa, E. Cífková, M. Holčapek, Retention behavior of lipids in reversed-phase ultrahigh-performance liquid chromatography–electrospray ionization mass spectrometry, Journal of Chromatography A, 1450 (2016) 76-85.

List of Students' Published Works

1/ E. Cífková, R. Hájek, M. Lísa, M. Holčapek, Hydrophilic Interaction Liquid Chromatography – Mass Spectrometry of (Lyso)Phosphatidic acids, (Lyso)Phosphatidylserines and Other Lipid Classes, J. Chromatogr. A 1439 (2016) 65-73.

2/ R. Hájek, R. Jirásko, M. Lísa, E. Cífková, M. Holčapek, Identification of Gangliosides in Biological Samples Using Hydrophilic Interaction Liquid Chromatography – Mass Spectrometry, Anal. Chem. 89 (2017) 12425–12432.

3/ R. Hájek, M. Lísa, M. Khalikova, R. Jirásko, E. Cífková, V. Student, D. Vrána, M. Matzenauer, L. Opálka, K. Vávrová, B. Melichar, M. Holčapek, HILIC/ESI-MS determination of gangliosides and other polar lipid classes in renal cell carcinoma and surrounding normal tissues, Anal. Bioanal. Chem. 410 (2018) 6585-6594.

4/ O. Peterka, R. Jirásko, M. Chocholoušková, D. Wolrab, R. Hájek, L. Kuchař, D. Vrána, M. Matzenauer, B. Melichar, M. Holčapek, Lipidomic comparison of exosomes and plasma by various mass spectrometry methods for kidney cancer patiens and control healthy, in press.