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Lipidomic analysis using the coupling of separation techniques and mass spectrometry

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Abstract

Living organisms have always been studied by scientific disciplines, and it is also valid at present. New modern approaches open new possibilities, which relates to the considerable expansion of „omics“ methods including genomics, proteomics, metabolomics, or glycomics. Lipidomics is the youngest subgroup of metabolomics focusing on the analysis of lipids in living organisms. Lipids are a large group of natural substances, which are contained in plants, fungi, bacteria, or in the body of animals, where they are responsible for essential life functions. Mass spectrometry is the most widely used technique in the qualitative and quantitative analysis of lipids.

The theoretical part of this thesis describes the basic knowledge about lipids, systematic nomenclature, and biological functions in living organisms. At the same time, the lipidomic analysis is described from sample preparation to data processing. A special attention is paid to the principles of chromatographic techniques associated with mass spectrometry for lipidomic analysis, and the advantages, disadvantages, and principles of qualitative and quantitative analysis are summarized. The review shows the potential of lipids for the diagnosis of various types of diseases and the benefits of derivatization procedures in lipidomic analysis.

The experimental part deals with the development of new methods for the analysis of lipids in body fluids by chromatographic techniques coupled with mass spectrometry. Lipidomic analysis follows two chromatographic approaches in this thesis, lipid species separation is achieved by reversed-phase liquid chromatography and lipid class separation by hydrophilic interaction chromatography and supercritical fluid chromatography. Lipid species separation is used for the qualitative analysis of human plasma by nonderivatization and derivatization approaches. On the other hand, the lipid class separation is used for the quantitative analysis of a large number of samples, and statistical tools are used to compare the lipid profiles of healthy volunteers and cancer patients.

Abstrakt

Studium živých organismů bylo vždy zájmem vědeckých oborů a v dnešní době tomu není jinak. S moderními přístupy se otevírají nové možnosti, což souvisí i se značným rozmachem tzv. „omics“ metod, kam patří například genomika, proteomika, metabolomika nebo glykomika. Lipidomika je nejmladším podoborem metabolomiky a zaměřuje se na analýzu lipidů v živých organismech. Lipidy jsou rozsáhlou skupinou přírodních látek, které jsou obsaženy v rostlinách, houbách, bakteriích nebo v těle živočichů, kde plní životně důležité funkce. Hmotnostní spektrometrie je nejpoužívanější technikou v kvalitativní i kvantitativní analýze lipidů.

V teoretické části této práce jsou popsány základní poznatky o lipidech, současné názvosloví a biologické funkce v živých organismech. Současně je vysvětlena lipidomická analýza od přípravy vzorků až po nástroje využívané ke zpracování naměřených dat. Zvláštní pozornost je věnována principům chromatografických technik a využití jejich spojení s hmotnostní spektrometrií v lipidomické analýze, kdy jsou kromě výhod a limitací shrnuty také zásady pro kvalitativní a kvantitativní analýzu. Literární rešerše poté ukazuje potenciál lipidů pro diagnostiku různých typů onemocnění a přínos derivatizačních postupů v lipidomické analýze.

V experimentální části je diskutován vývoj nových metod pro analýzu lipidů v tělních tekutinách s využitím spojení chromatografických technik a hmotnostní spektrometrie. V této práci jsou použity dva přístupy využívané v lipidomické analýze, separace lipidů dle lipidových druhů pomocí kapalinové chromatografie s obrácenými fázemi a separace dle lipidových tříd pomocí hydrofilní interakční chromatografie a superkritické fluidní chromatografie. Separace lipidů dle lipidových druhů je aplikována pro kvalitativní popis lidské plazmy s využitím klasického i derivatizačního přístupu, naopak metody založené na separaci dle lipidových tříd jsou využity pro kvantitativní analýzu velkého množství vzorků a pomocí statistických nástrojů jsou porovnávány lipidové profily zdravých jedinců a pacientů s diagnostikovanou rakovinou.

Keywords

Lipids, oncolipidomics, liquid and supercritical fluid chromatography, mass spectrometry, derivatization.

Klíčová slova

Lipidy, onkolipidomika, kapalinová a superkritická fluidní chromatografie, hmotnostní spektrometrie, derivatizace.

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Introduction

1 Lipidomics

Lipidomics is a subgroup of metabolomics aiming at the analysis of lipid species in biological systems. Lipids are a diverse group of biomolecules, which are most often described as fatty acids, their derivatives, and substances that are biosynthetically or functionally related to these compounds [1]. LIPID MAPS is the most extensive database of lipid describing over 44,500 of molecules, which are divided into eight lipid categories (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterols, prenols, polyketides, and saccharolipids) shown in Figure 1. The individual categories are further divided into lipid classes and subclasses [2]. Since lipidomics is a young discipline, it is necessary to introduce precisely defined terms for a uniform nomenclature. The first nomenclature was established by Fahy et al. in 2005 [2] followed by several updates and the newest published by Liebisch et al. in 2020 [3].

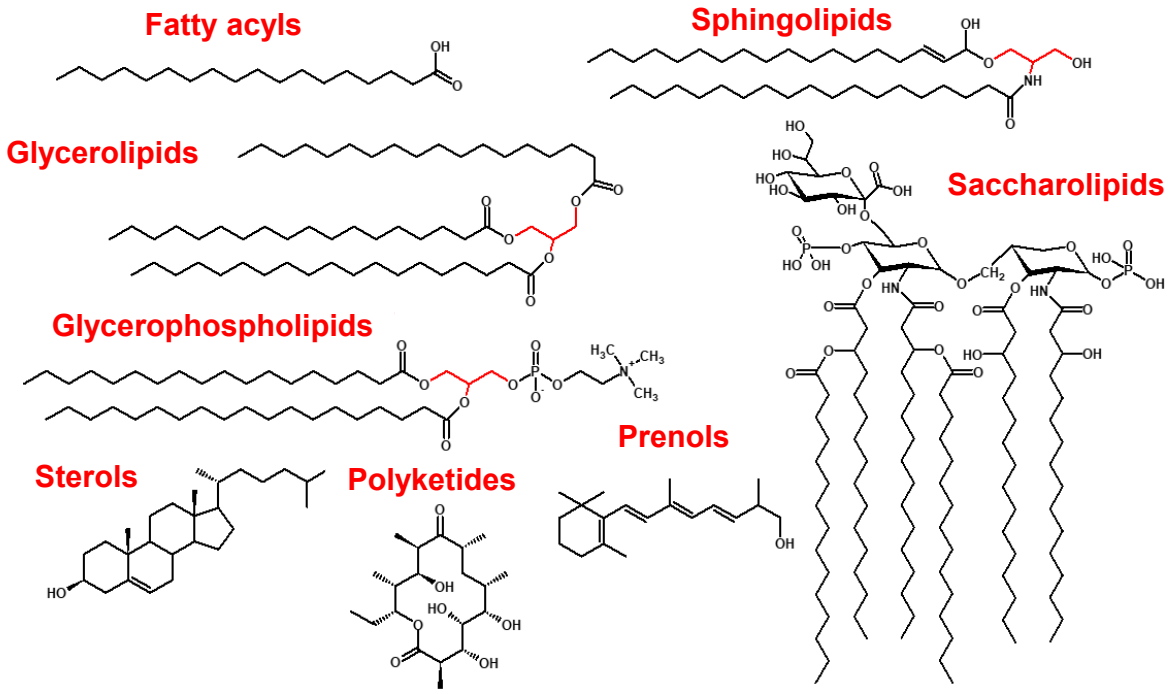

Figure 1: The LIPID MAPS classification with examples of molecules representing individual lipid categories.

1.1 Biological function of lipids

Lipids play important roles in cells and have various biological functions, such as energy storage, membrane components and precursors for metabolic processes. The formation of a phospholipid bilayer is probably the most famous function of lipids, where the main components are phospholipids, sphingolipids, and cholesterol. The composition of fatty acyls and polar/ionic head group play important roles in viscosity, rigidity, and shape of membrane. Glycerolipids are presented mainly as energy storage, which are hydrolyzed by various lipases producing free fatty acids and glycerol, and energy is released by metabolic processes in the form of adenosine triphosphate. However, diacylglycerols and monoacylglycerols are also signaling molecules, such as some phospholipids, sphingolipids, and fatty acyls [4]. The biological functions are summarized in Table 1.

Table 1: The main biological functions of individual lipid classes.

Lipid categories	Membrane components	Energy storage	Signaling
Fatty acyls	–	NEFA, AcylCAR	NEFA, AcylCAR, NAE, oxylipins
Glycerolipids	–	TG, DG, MG	DG, MG
Glycerophospholipids	PC, PE, PI, PG, PA, PS, CL	–	*Lyso*-forms
Sphingolipids	SM, HexCer, SHexCer, gangliosides	–	Cer, SPB, S1P
Sterols	Cholesterol	–	–

Lipids are an important part of the human body performing various functions and concentration changes can indicate various diseases. The most routine analyzes for diagnosis are determination of cholesterol, cholesterol HDL (high-density lipoprotein), total triacylglycerols, bile acids, and lipid-based hormones [5]. While signaling lipids show the great potential for biomarkers research for serious diseases, such as cancer [6], neurodegenerative diseases [7], cardiovascular diseases [8], and diabetes mellitus [9].

1.2 Lipidomic analysis

Lipids are a large group of substances that need to be considered in lipidomic analysis. The preanalytical part (collection of samples and their storage) is an important part of the whole analysis and it is necessary to follow protocols to avoid changes in lipid composition due to oxidation or enzymatic reactions. The lipidomic extraction is selected based on the character of analyzed lipids, with liquid-liquid extraction being the most common.

1.2.1 Sample preparation

The principle of liquid-liquid extraction is based on the distribution of the analyte between 2 immiscible phases. The Folch [10] and Bligh-Dyer [11] are chloroform-based extractions, which are the oldest method, but still widely used methods. In order to reduce halogenated solvents, new processes have been developed, such as BuMe [12], BuHe [13], and MTBE [14] methods. In addition to the already mentioned two-phase extractions, three-phase [15] and single-phase [16] extractions have also been described. Method of protein precipitation by organic reagents, such as acetonitrile, acetone, ethanol, methanol, and mixtures BuOH/MeOH or MeOH/MTBE/$CHCl_3$, has become popular due to speed and simplicity [17]. Solid phase extraction is a selective technique based on the different affinity of substances to the sorbent. Selection of sorbent and the elution solvent are the critical part of the technique. This method works with small volumes of solvents and allows not only concentration of the sample, but also possible fractionation [18]. Supercritical fluid extraction (SFE) uses fluid in a supercritical condition, of which viscosity is similar to gas and its density to liquid. It is a fast, selective, and efficient extraction, which is controlled mainly by temperature and pressure. Since carbon dioxide is cheap, chemically stable, non-toxic, non-flammable, and its critical values are relatively low, it is the most widely used fluid for SFE. Because the polarity of CO_2 is close to hexane, the extraction is mainly used for compounds with low polarity, but the addition of modifiers is extended to substances with higher polarity [19].

1.2.2 Lipidomic analysis by mass spectrometry

Mass spectrometry is a key technique for lipid analysis and three main approaches in lipidomic analysis are direct infusion MS analysis (called shotgun), separation techniques coupled to MS, and desorption ionization techniques MS approaches (mass spectrometry imaging). Mass spectrometry coupled with separation techniques enable the identification and quantification of a large number of lipids from various lipid categories, due to the separation of isomeric and isobaric forms of lipids and lipid class separation and lipid species separation are the main two approaches [20,21].

1.2.2.1 Lipid class separation

Lipid class separation methods separate lipid according to polar/ionic head group, which is achieved by hydrophilic interaction chromatography (HILIC) for polar lipids, normal phase liquid chromatography for nonpolar lipids, and supercritical fluid chromatography (SFC) for polar and nonpolar lipids. This approach enables easier identification and quantification, due to the coelution of all lipid species from one lipid class in the same peak. One internal standard per lipid class is acceptable for quantification, due to the same matrix effects caused by the coelution of analytes and internal standard [20,21].

1.2.2.2 Lipid species separation

Lipid species separation methods separate lipids according to the composition of acyl chain(s). The retention time is increasing with the increase of equivalent carbon number, which corresponds to the length of acyl chain and the number of double bond(s). Lipid species separation is achieved by reversed phase liquid chromatography (RP-LC) and non-aqueous reversed-phase (NARP) LC. RP-LC is the most widespread chromatographic mode in the lipidomics research enabling separation of various isomeric forms. Quantitative analysis is more complicated in this case, because IS does not coelute with analytes. The best way for absolute quantification is isotopic labelled internal standard per analyte, but it is impossible in omics techniques due to large

number of compounds. Alternative is using more IS covering the whole retention window of the lipid class [20,21].

1.2.3 Derivatization

Derivatization is a chemical reaction that changes physico-chemical properties for better separation, peak shape, and higher sensitivity of detection. Implementation of chromophore or fluorophore are the most common reaction, but increased ionization efficiency, change ion polarity, and map metabolic pathways using isotopically labeled reagents are the main reasons using in mass spectrometry. Pooling of isotopic non-labelled and labelled derivatives is a new approach in lipidomics, which leads to a high confident level of identification and more accurate quantitative analysis. The principle of analysis is shown in Figure 2. Moreover, the lipids contain many functional groups that can be derivatized, such as hydroxyl group, primary and secondary amino groups, carboxylic group, and phosphate group and large number of papers were published. This work is focused on derivatization for liquid and supercritical fluid chromatography [22].

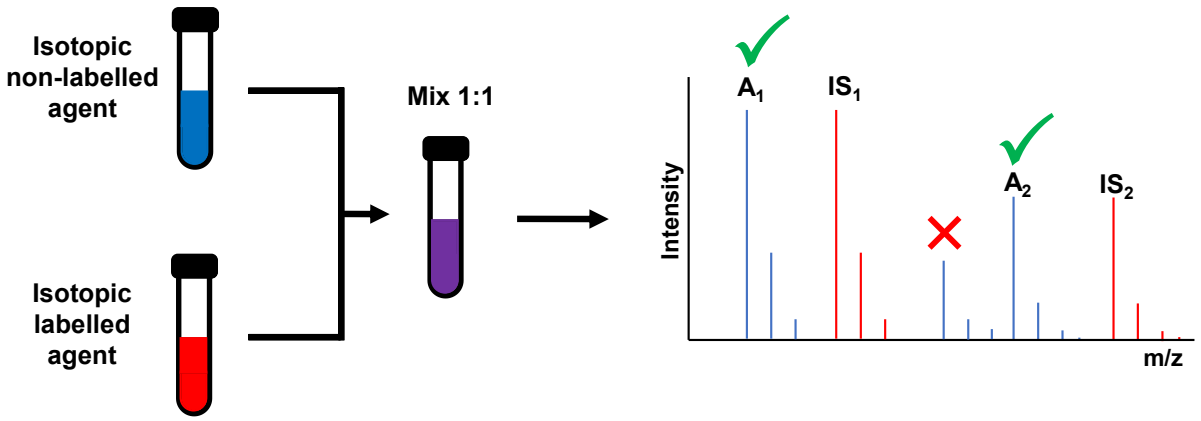

Figure 2: Principle of identification using isotopically non-labelled and labelled derivatization agents.

2 Materials and methods

The whole range of materials was used during my Ph.D. study, which is described in the published paper. Therefore, only the methods are summarized in this part.

2.1 Lipid species separation

Lipid extracts were prepared using protein precipitation by organic solvents. The small volume of human plasma (10 µL) was mixed with organic solvent (ACN or mixture of BuOH/MeOH), sonicated, centrifuged, and filtered. In case of derivatization approach, the extracts were redissolved in 335 µL of pyridine (1:10 in ACN, *v/v*), and 120 µL (1:10 in ACN, *v/v*) of benzoyl chloride as the derivatization agent was added. The reaction mixture was slowly stirred at ambient temperature for 60 minutes. The reaction was stopped by applying a modified Folch lipid extraction protocol.

We used Agilent 1290 Infinity LC series connected to high-resolution mass spectrometer Xevo G2-XS QTOF (Waters). Acquity UPLC BEH C18 column (150 mm × 2.1 mm, 1.7 µm, Waters) was employed with the injection volume 1 µL. The gradient elution using phase A ACN/H_2O (3:2, *v/v*) and phase B IPA/ACN (9:1, *v/v*), both phases containing 8 mM, resp. 5 mM ammonium formate and 0.1% formic acid, was set for the separation of lipid species. The total run time including the equilibration is 25 min.

2.2 Lipid class separation

The biological samples (human plasma, serum, and exosomes) were spiked by IS-Mix before the extraction. The modified Folch extraction protocol was used, where 3 mL of mixture chloroform/MeOH (2:1, *v/v*) were added. The mixture was homogenized for 15 min at 40 °C in an ultrasonic bath. Then, 600 µL of 250 mM ammonium carbonate buffer was added. After the centrifugation was collected organic layer and evaporated. The residue was dissolved in 500 µL $CHCl_3$/MeOH (1:1, *v/v*) and for UHPSFC and UHPLC analysis were samples diluted 1:5 (*v/v*) with $CHCl_3$/MeOH (1:1, *v/v*) before the analysis.

We used UHPSFC Acquity UPC^2 (Waters) connected to the hybrid quadrupole time-of-flight mass spectrometer Synapt G2-Si (Waters). For the separation of lipid

classes, Acquity BEH UPC^2 (100 mm × 3 mm, 1.7 µm, Waters) column was employed, the column temperature 60 °C was set, and the injection volume 1 µL. The linear gradient of phase A ($scCO_2$) and phase B (MeOH with 30 mM ammonium acetate and 1% H_2O) was set with the flow rate 1.9 mL/min. The make-up solvent (the same composition as phase B) with flow rate 0.25 mL/min was mixed with eluent for higher ionization efficiency. The total run time including the equilibration is 8 min.

HILIC-UHPLC was performed on an Agilent 1290 Infinity series UHPLC system connected to Xevo G2-XS QTOF (Waters). The column Viridis BEH Waters (100 mm × 3 mm, 1.7 µm) with the column temperature of 40 °C, the flow rate 0.5 mL/min, and the injection volume 1 µL was used. The linear gradient of phase A (ACN/H_2O, 96/4, *v/v*) and phase B (ACN/H_2O, 2/98, *v/v*) containing 8 mM of ammonium acetate in both phases. The total run time including the equilibration is 10.5 min.

3 Results and discussion

The aim of the Ph.D. thesis is lipidomic analysis using various chromatographic techniques connected to mass spectrometry. I used lipid species separation approach (RP-UHPLC) and lipid class separation approach (UHPSFC and HILIC-UHPLC) for the analysis of human body fluids (plasma and serum) and extracellular vesicles (exosomes). The methods are used for qualitative and quantitative analysis focusing on the most abundant lipid classes in human lipidome, such as MG, DG, TG, CE, Cer, PC, LPC, PE, and SM. In some cases, shotgun analysis and matrix-assisted laser desorption/ionization are applied as references method.

3.1 Lipid species separation

RP-UHPLC/MS method was developed with the aim to unambiguously identify a large number of lipid species from multiple lipid classes in human plasma. The optimized RP-UHPLC/MS method employs the C18 column with sub-2 μm particles with the total run time of 25 min. Human plasma was analyzed by our RP-UHPLC/MS method using the high confidence level of identification based on *m/z* accuracy (lower than 5 ppm) in positive and negative ion mode, tandem mass spectrometry, and retention dependences (RT *vs.* DB and RT *vs.* CN). In total, 503 lipid species representing 26 lipid classes were identified in human plasma NIST SRM 1950 and we summarized the characteristic ions in MS and MSMS for all identified lipid classes. In comparison with lipid class separation approaches, we identified many times more lipid species, due to the separation of isomeric and isobaric forms. Moreover, we determined fatty acyls composition by MSMS specters using the ratio of fragments (glycerolipids and glycerophospholipids) and characteristic fragments of sphingoid bases (sphingolipids). The paper describes a large number of lipids in human plasma, and the plan will be focused on the quantitative analysis of these compounds **(Paper no. 1)**.

The next paper builds on the previous work, which uses the same separation methods, but sample preparation is different using derivatization of lipids after protein precipitation. The aim of this work is increased extraction efficiency for polar lipids and sensitivity for nonpolar lipids. Benzoyl chloride is the best candidate for derivatization

agent, due to safety, availability, and high reactivity, which is confirmed by reaction with standards from 13 lipid classes. The derivatives are characterized by MS and MSMS using high resolution mass spectrometry. The character of lipids is more nonpolar after derivatization, which leads to increase retention time (except LPC and PE-P). The derivatization process was optimized using spiked human plasma by IS-Mix. The molar ration 4:1 (pyridine/benzoyl chloride) reacts one hour at ambient temperature provide the best results. The repeatability and reproducibility were investigated by one, resp. two operator(s) with multiple independent reactions. The relative standard deviation (RSD) is lower than 15 % (except for low abundant lipid species investigated by the operator without previous derivatization experience). Stability of derivatives were determined by periodical measurements during one month period and five freeze/thaw cycles stored at -80 °C. The advantages of derivatization approach are shown using parameters calculated or determined from calibration curves (linear range, limit of detection, and slope of curve). The spiked human plasma (10 concentration levels) by IS-Mix including 22 internal standards from 12 lipid classes are used for the construction of the calibration curves. Linear range is significantly wider for MG, DG, SPB, and cholesterol and the sensitivity is increased 2 to 10-fold for almost all investigated lipid classes and even more than 100-fold for MG. The new method decreases the limit of detection 9-fold for MG, 6.5-fold for SPB, and 3-fold for MG. Finally, human plasma was analyzed by derivatization method and 169 derivatized lipids from 11 lipid classes are reported using the high confidence level of identification. In comparison with other RP-UHPLC/MS without derivatization step, we report more lipid species for monoacylglycerol, diacylglycerols, and sphingoid bases. Moreover, our method enables fatty acyls in positive ion mode compared to other methods forced to switch to the negative ion mode and detects the molecular ion of cholesterol. The next step is applied derivatization method for targeted lipidomic analysis using characteristic scans (precursor ion scan, scan of neutral losses, and multiple reaction monitoring) using isotopic labelled derivatization agent **(Paper no. 2)**.

3.2 Lipid class separation

The aim of this part is to develop a quantitative method for the analysis of hundreds lipid extracts. The UHPSFC/MS and HILIC-UHPLC/MS methods were optimized and validated following EMA and FDA recommendations for TG, DG, MG, Cer, PC, LPC, PE, and SM. Moreover, we investigated the composition of internal standard mixture corresponding to the concentration of lipids in human plasma/serum. The new IS-Mix contains IS for more lipid classes and two IS per lipid class for almost all lipid classes. We also optimized the extraction procedure and modified Folch extraction is the best choice for investigated lipid classes. The methods were used for quantitative analysis of human plasma NIST SRM 1950. Both methods are compared using correlation graphs and small differences are observed for glycerolipids, probably due to the quantification of these classes in the void volume. Comparison with reference values shows comparable results. The comparison of biological materials (plasma *vs.* serum) shows the same lipidomic composition, but serum reports higher concentration of lipids ca. 20 – 30 %. The results confirm the potential of UHPSFC/MS and HILIC-UHPLC/MS methods for high-throughput lipidomic analysis, and strengths and weaknesses are described. Shorter run time (8 *vs.* 10.5 min) and separation of nonpolar and polar lipids are advantages of UHPSFC/MS method, but HILIC-UHPLC/MS method shows higher sensitivity for polar lipids **(Paper no. 3)**.

The high-throughput analysis produces large number of data files and data processing consumes time of the operator. For this reason, we implement the Excel macroscript called LipidQuant 1.0. The software enables automatic identification and quantification for lipid class separation approaches. The identification is based on the comparison between experimental and theoretical *m/z* values with mass deviation set by operator. The database includes 23 lipid classes (1 470 lipid species) for positive ion mode and 24 lipid classes (1 999 lipid species) for negative ion mode, but the database can be easily modified by operator. Quantitative values are calculated as the ratio of intensity of analyte and internal standard multiplied by concentration of IS. The isotopic correction of II. type is applied automatically by software. The using of LipidQuant 1.0 is demonstrated on the study investigated differences between males and female lipidome. We focused on 8 lipid classes (CE, TG, DG, MG, Cer, PC, LPC, and SM) in

positive ion mode analyzed by UHPSFC/MS method. In total, we quantified 152 lipids and the data are visualized by principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). The models show the separation of individual gender and quality control samples are focused close to one point confirming the quality of data sets. Upregulation of glycerophospholipids in women and glycerolipids in men have been discovered by S-curve and visualized by box plots. However, the aim of this article is the demonstration of software for a large number of samples, not separation of genders based on lipidome **(Paper no. 4)**.

Lipid class separation methods combined with automated data processing were used for monitoring plasma lipidomic profile during one year. The samples have been obtained from 99 volunteers at three time points separated by six-month intervals. We monitored 171 lipid species from 8 lipid classes by UHPSFC/MS method and 122 lipid species from the 4 lipid classes by HILIC-UHPLC/MS method. The data were normalized using plasma NIST SRM 1950, due to changes of mass spectrometry condition influence in this long period. Results indicate that the concentrations of SM, LPC, and Cer are relative stable over the period of one year, whereby glycerolipids are prone to concentration changes over time. At the same time, we investigated the influence of blood collection tubes using for isolation of plasma (heparin and EDTA) and serum. The results show no significant changes, only higher concentration of lipids was determined in serum, which corresponds with results of previous work. Comparison of two different collection places shows a comparable lipidomic profile of CE, SM, PC, LPC, and Cer, but differences for glycerolipids probably caused by preclinic factors. The significant differences are indicated in the comparison of our cohort (Czech Republic) with plasma NIST SRM 1950 (USA), which shows different lipidomic composition of human plasma based on geographic origin **(Paper no. 5)**.

In the next study, UHPSFC/MS, HILIC-UHPLC/MS, and MALDI-HRMS methods are used for the characterization of exosomes, which were isolated from human plasma of 12 volunteers using commercial kit. In total, we quantified 244 lipid species in exosomes and 191 lipid species in original plasma and the absolute concentrations are expressed in nmol/mL of processed plasma. Results show five times higher concentration of lipids in plasma, because plasma has other sources of lipids in addition

to exosomes. Relative concentrations clearly illustrate the enrichment of specific lipids within one class over other lipid species from the same class. Higher concentration of glycerolipids (TG, DG, and MG) in exosomes could be caused by the adhesion of lipoproteins to exosomes associated with TG transmission or the role of exosomes as a source of substrates and enzymes for distant metabolic sites. Higher concentration of DG could be caused the degradation of TG or action of phospholipases, which correlates with lower concentration of PC. Various concentrations of phospholipids (PC and LPC) and sphingolipids (SM and Cer) could be caused by different composition of membranes, which correlated with upregulated saturated LPC and downregulated LPC 18:2. Since exosomes are still underexplored nanoparticles, our explanations of dysregulation are rather hypotheses based on the cases described in published papers. However, we show a difference in lipid composition of plasma and exosomes and demonstrate the potential of exosomes for further studies of cellular communication **(Paper no. 6)**.

3.3 Oncolipidomics

Oncolipidomics is a new term, which summarizes the quantitative lipidomic analysis using mass spectrometry in cancer research. The first part of the review summarizes information about lipids, biological functions, sample preparation, and biological materials using in clinical applications. The second part describes lipidomic analysis using mass spectrometry with direct infusion, coupling with liquid-phase separation techniques, or coupling with desorption ionization techniques using for mass spectrometry imaging. We summarize the general principles of techniques and strengths and weaknesses for qualitative and quantitative analysis. The last part focuses on dysregulation lipids reported in 12 cancer types by analyzing of tissues and human body fluids (plasma, serum, and urine). We summarize results of 74 studies and the most frequently dysregulated lipids were LPC and PC, which demonstrates the potential of lipidomic analysis for cancer detection **(Paper no. 7)**.

The next study compares the lipidomic profile of healthy subjects and patients with pancreatic cancer (PDAC), whereas the study is divided into three phases (discovery, qualification, and verification). The aim of the discovery phase was the

differentiation of PDAC patients and healthy controls by UHPSFC/MS, DI-MS (LR), and MALDI-MS methods. In total, we quantified 364 samples (262 PDAC and 102 healthy controls) by UHPSFC/MS and DI-MS (LR) and 64 samples by MALDI-MS, due to the complexity of sample preparation. The differences are discovered by statistical tools and visualization by multivariate data analysis (MDA). The PCA model shows a partial separation, which is increased by dividing the model by gender. The predicted response values for training and validation sets obtained from OPLS-DA models are higher than 95 %. The second phase verifies the results from laboratories in Czech Republic, Germany, and Singapore, whereas all laboratories analyzed the same samples using methods established in that laboratory. Lipid extracts were prepared by Folch, Bligh-Dyer, and protein precipitation by the mixture of BuOH/MeOH. Then, 554 extracts were analyzed using UHPSFC/MS, DI-MS (LR), DI-MS (HR), and RP-UHPLC/MS. Data are normalized using human plasma NIST SRM 1950 and RSD is lower than 40 %, which is acceptable due to using various types of sample preparation, methods, and internal standards. Moreover, the trends of dysregulated lipids are the same for all three laboratories. In the verification phase, we analyzed 830 samples from four blood collection sites by UHPSFC/MS. The samples are divided into data sets (training and validation), whereas validation set is used for prediction and sensitivity, specificity, and accuracy are higher than 80 %. The most dysregulated lipids (SM 41:1, SM 42:1, Cer 41:1, Cer 42:1, SM 39:1, LPC 18:2, a PC O-36:3) are identified using statistical tools and multivariate data analysis. Our method also enables the separation of PDAC and patients with pancreatitis, because the lipidomic profile is similar to healthy volunteers. Lipidomic profiling is compared with other methods, such as biomarker CA 19-9 and CancerSeek. The ROC curve shows the best result for the combination of lipidomic profiling and biomarker CA 19-9 followed by lipidomic profiling, CancerSeek test, and biomarker CA 19-9. On the other side, lipidomic profiling reports lower values of specificity, but the advantage is sensitivity for early stages **(Submitted paper no. 1)**.

Differences between healthy volunteers and cancer patients are investigated also in the last study. Quantitative analysis of 289 cancer samples (119 kidney, 103 breast, and 67 prostate) and 192 healthy controls were performed by UHPSFC/MS and DI-

MS (LR). Samples are divided into two groups again, and the validation set is also used for prediction using OPLS-DA models. The average values of sensitivity, specificity, and accuracy are higher than 85 % for all parameters. The most dysregulated lipids are CE 16:0, Cer 42:1, LPC 18:2, PC 36:2, PC 36:3, SM 32:1, and SM 41:1 identified by statistical tools and MDA. Based on statistical parameters (fold change, p-value, VIP value, and Bonferroni correction), the numbers of lipid species in models are decreased and the influence on the prediction capability of statistical models is investigated. The number is reduced from original 138 lipid species to 91, 23, and finally to 7 potential biomarkers for these three cancer types. Reduction of lipid species in the model negatively affects sensitivity but has minimal effect on specificity. At the same time, we investigate differences between individual cancer types (kidney *vs.* prostate in male and breast *vs.* kidney in females), which are only partially separated in OPLS-DA models **(Paper no. 8)**.

Conclusions

The Ph.D. thesis is focused on lipidomic analysis by separation techniques coupled to mass spectrometry. I used lipid class (UHPSFC and HILIC-UHPLC) and lipid species (RP-UHPLC) separation approaches for quantitative and qualitative analysis of plasma, serum, and exosomes.

Lipid species separation approach was applied to identify the maximum number of lipids in human plasma. Lipid species were identified based on the high confidence level of identification (mass accuracy in positive and negative ion modes, tandem mass spectrometry, and retention dependences) by RP-UHPLC/MS. Moreover, a new derivatization method was developed with benzoyl chloride as the derivatization agent, which increased the sensitivity for almost all lipid classes and significantly decreased LOD of nonpolar lipids. The high confidence level of identification was used also in this case. In total, more than 500 lipid species representing 27 lipid classes were identified in human plasma by both methods.

Lipid class separation methods were developed for high-throughput quantitative analysis of human body fluids and were fully validated for MG, DG, TG, CE, Cer, SM, PC, and LPC following EMA and FDA recommendations. In total, over 150 lipid species were quantified from 8 lipid classes in human plasma. Combination of high-throughput methods and our laboratory software LipidQuant 1.0 enabled the measurement of a large number of samples, which allowed lipidomic profiling of healthy volunteers and cancer patients (pancreas, breast, kidney, and prostate). Statistical analysis and multivariate data analysis identified the most dysregulated lipids (CE 16:0, Cer 42:1, Cer 41:1, LPC 18:2, PC 36:2, PC 36:3, PC O-36:3, SM 32:1, SM 41:1, and SM 39:1) for these four types of cancer.

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List of publications

Published papers:

1. Z. Vaňková, **O. Peterka**, M. Chocholoušková, D. Wolrab, R. Jirásko, M. Holčapek. Retention dependences support highly confident identification of lipid species in human plasma by reversed-phase UHPLC/MS. Analytical and Bioanalytical Chemistry, 2021 (in press).
2. **O. Peterka**, R. Jirásko, Z. Vaňková, M. Chocholoušková, D. Wolrab, J. Kulhánek, F. Bureš, M. Holčapek. Simple and Reproducible Derivatization with Benzoyl Chloride: Improvement of Sensitivity for Multiple Lipid Classes in RP-UHPLC/MS. *Analytical Chemistry*, 2021 (in press).
3. D. Wolrab, M. Chocholoušková, R. Jirásko, **O. Peterka**, M. Holčapek. Validation of lipidomic analysis of human plasma and serum by supercritical fluid chromatography–mass spectrometry and hydrophilic interaction liquid chromatography–mass spectrometry. *Analytical and Bioanalytical Chemistry*, 2020, 412, 10, 2375-2388.
4. D. Wolrab, E. Cífková, P. Čáň, M. Lísa, **O. Peterka**, M. Chocholoušková, R. Jirásko, M. Holčapek. LipidQuant 1.0: automated data processing in lipid class separation - mass spectrometry quantitative workflows. *Bioinformatics,* 2021 (in press).
5. D. Wolrab, M. Chocholoušková, R. Jirásko, **O. Peterka**, V. Nováková Mužáková, B. Melichar, M. Holčapek. Effect of blood collection tubes and collection time on lipidomic profiles determined by UHPSFC/MS and HILIC-UHPLC/MS. *Analytica Chimica Acta*, 2020, 1137, 74-84.
6. **O. Peterka**, R. Jirásko, M. Chocholoušková, L. Kuchař, D. Wolrab, R. Hájek, D. Vrana, O. Strouhal, B. Melichar, M. Holčapek. Lipidomic characterization of exosomes isolated from human plasma using various mass spectrometry techniques. *Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids*, 2020, 1865, 158634.

7. D. Wolrab, R. Jirásko, M. Chocholoušková, **O. Peterka**, M. Holčapek, Oncolipidomics: Mass Spectrometric Quantitation of Lipids in Cancer Research, *TrAC Trends in Analytical Chemistry,* 2019, 120, 115480.
8. D. Wolrab, R. Jirásko, **O. Peterka**, J. Idkowiak, M. Chocholoušková, Z. Vaňková, K. Hořejší, I. Brabcová, D. Vrána, H. Študentová, B. Melichar, M. Holčapek. Plasma Lipidomic Profiles of Kidney, Breast, and Prostate Cancer Patients Differ from Healthy Controls. *Scientific Reports*, 2021 (in press).

Submitted paper:

1. D. Wolrab, R. Jirásko, E. Cífková, M. Höring, D. Mei, M. Chocholoušková, **O. Peterka**, J. Idkowiak, T. Hrnčiarová, L. Kuchař, R. Ahrends, R. Brumarová, D. Friedecký, G. Vivo-Truyols, P. Škrha, J. Škrha, R. Kučera, B. Melichar, G. Liebisch, R. Burkhardt, M. R. Wenk, A. Cazenave-Gassiot, P. Karásek, I. Novotný, K. Greplová, R. Hrstka, M. Holčapek. Lipidomic profiling of human serum enables detection of pancreatic cancer. *Nature Communication*. 2021. 2. revision. Preprint available at https://www.medrxiv.org/content/10.1101/2021.01.22.21249767v1.full.pdf.