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Quantitative lipidomics: High-throughput analysis of clinical samples of body fluids

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References

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Abstract

Lipidomics is a subgroup of metabolomics, which is the last step of the omics cascade closest to the organism function, and therefore the analysis of metabolites (including lipids) can provide an important information about ongoing processes in the human body, such as diseases or disorders reflected in dysregulations of lipid concentrations. The main goal of this Ph.D. thesis is the development of high-throughput methods for the quantitation of lipids in biological samples using the following mass spectrometry (MS) based strategies: lipid species separation and lipid class separation, which have own requirements for the lipid quantitation mainly based on the selection and the number of internal standards (IS). The lipid species separation is applied for the analysis of oxylipins using a reversed-phase ultrahigh-performance liquid chromatography (RP-UHPLC) coupled to tandem mass spectrometry with prior solid phase extraction from biological samples. The lipid class separation is used for the comprehensive quantitation of multiple lipid classes including monoacylglycerols, diacylglycerols, triacylglycerols, cholesteryl-esters, ceramides, sphingomyelins, phosphatidylcholines, and lysophosphatidylcholines. The lipid class separation approach is achieved by hydrophilic interaction ultrahigh-performance liquid chromatography (HILIC-UHPLC) or ultrahigh-performance supercritical fluid chromatography (UHPSFC) coupled to mass spectrometry. Advantages and limitations of both approaches for the lipidomic quantitation are discussed including the method validation, the use of quality control samples, and the data normalization using NIST SRM 1950 Reference Material human plasma. Finally, all developed analytical methods have been applied for sets of clinical samples of cancer patients and healthy controls followed by multivariate data analysis to distinguish both groups.

Abstrakt

Lipidomika je podskupinou metabolomiky, která je jako poslední krok omické kaskády nejblíže k funkci organismu, proto analýza metabolitů (zahrnující lipidy) může poskytnout důležité informace o probíhajících procesech v lidském těle spojené se změnami koncentrací lipidů. Cílem této práce je vývoj vysokokapacitních metod pro kvantifikaci lipidů v biologických vzorcích pomocí metod chromatografie spojené s hmotnostní spektrometrií: separace jednotlivých lipidů a separace tříd lipidů, které s sebou přináší vlastní nároky na kvantitu převážně založené na výběru a počtu interních standardů. Separace jednotlivých lipidů je aplikována na analýzu oxylipinů pomocí ultravysokoučinné kapalinové chromatografie v systémech s obrácenými fázemi spojené s tandemovou hmotnostní spektrometrií, kdy k přípravě biologických vzorků je nutná extrakce na pevné fázi. Separace tříd lipidů je použita pro stanovení kvantity několika lipidových tříd zahrnující monoacylglyceroly, diacylglyceroly, triacylglyceroly, cholesteryl-estery, ceramidy, sfingomyeliny, fosfatidylcholiny a lysofosfatidylcholiny. Separace tříd lipidů je možné dosáhnout pomocí hydrofilních interakcí ultravysokoučinné kapalinové chromatografie nebo ultravysokoučinné superkritické fluidní chromatografie spojených s hmotnostní spektrometrií. Výhody a limitace obou přístupů pro lipidomickou kvantifikaci jsou popsány spolu s validací metody, sledováním kontroly kvality měření a normalizací dat pomocí referenčního materiálu lidské plazmy NIST SRM 1950. Všechny vyvinuté metody byly nakonec použity pro měření série klinických vzorků pacientů s rakovinou a zdravých dobrovolníků s následnou vícerozměrnou statistikou analýzou dat pro rozlišení obou skupin.

Keywords

lipidomics; mass spectrometry; ultrahigh-performance liquid chromatography; ultrahigh-performance supercritical fluid chromatography; quantitation; quality control; validation; biological samples

Klíčová slova

lipidomika; hmotnostní spektrometrie; ultravysokoúčinná kapalinová chromatografie; ultravysokoúčinna superkritická fluidní chromatografie; kvantita; kvalita kontroly; validace; biologické vzorky

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Introduction

1. Lipidomics

Lipids are biomolecules with a high structure diversity. They differ in their various combinations of fatty acyls on backbone structures, such as glycerol or sphingoid base. Lipidomics is a study of lipids in cells or organism including a study about their functions and interactions with proteins, metabolites, or other lipids [1].

Lipids are the major components of membranes of organelles and cells, where lipids do not behave as single compounds, but mainly as a group of molecules. So, it is very important to analyze lipids comprehensively and quantitatively to understand their role in human body. The lipids are relatively small signaling and hydrophobic molecules, which may reflect the metabolic state of the organism and diseases, such as cancer, atherosclerosis, Alzheimer´s disease, obesity, heart diseases, etc. [2].

LipidMaps introduced a Lipid classification system in 2005, dividing lipids into eight lipid categories: fatty acyls (FA), glycerolipids (GL), glycerophosholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PL), saccharolipids (SL), and polyketides (PK) [3].

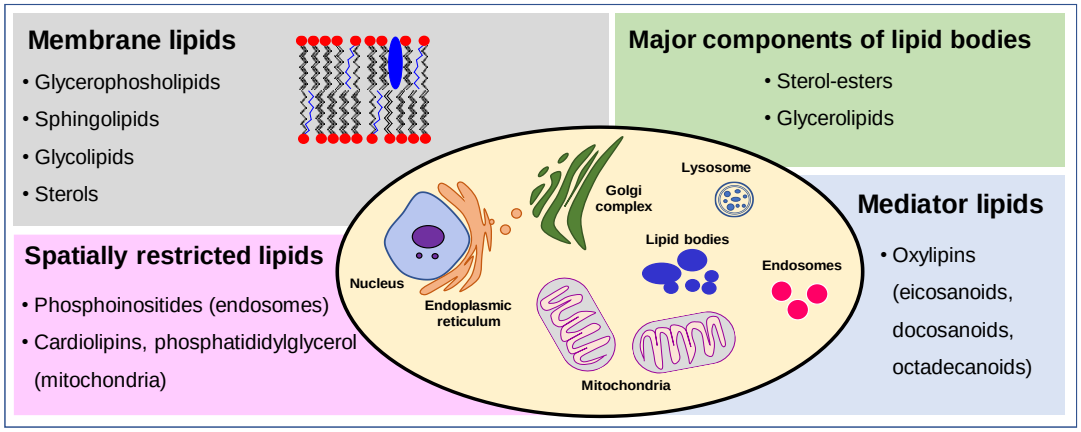

Figure 1 Importance of lipid classes in human body.

2. Lipidomic analysis

There are many steps, which influence the lipidomic analysis and it is very important to consider them. Preanalytic part is crucial for final evaluation and includes the study design, the type and description of samples together with clinical data, collection of samples, and their storage. Of course, other parts included in the lipidomic analysis are even more essential, such as quantitative

results, sample preparation (extraction), and sample and quality control (QC) measurements, to get reliable data [4].

Plasma and serum are body fluids, which are the most common biological samples for the analysis of human health state. Generally, lipids can be determined in all common biological matrices [5], such as cell lines, tissues [6-8], urine, cerebrospinal fluid, saliva [9], extracellular vesicles (exosomes) [10, 11], and capillary blood.

2.1 Lipid extraction

Liquid-liquid extraction is commonly used for the sample preparation in the lipidomic analysis. The main goal of the sample preparation protocol is to get analytes of interest and minimalized the complexity of the sample matrix, i.e., removing proteins [4]. Chloroform/methanol (2:1, *v/v*) based extractions are the most common protocols in lipidomic research: the lipid extraction introduced by Folch [12] or Bligh-Dyer using a mixture of chloroform/methanol at vice versa ratio (1:2, *v/v*) [13], both followed by addition of water. Other commonly used liquid-liquid extractions are based on addition of mixture of methyl *tert*-butyl ether (MTBE)/methanol followed by the addition of water [14], or the mixture of butanol-methanol (BUME) followed by the addition of heptan/ethyl acetate mixture and 1% of acetic acid in water [15]. Protein precipitation is used in the lipidomic research as well, using one solution like methanol or a mixture of solutions (for example butanol/methanol), what is one-phase extraction without any purification step typical for two-phase extraction [5]. To get higher selectivity of extraction, solid-phase extraction (SPE) is needed using pure silica or octadecylsilica cartridges depending on the nature of lipids of interest [16].

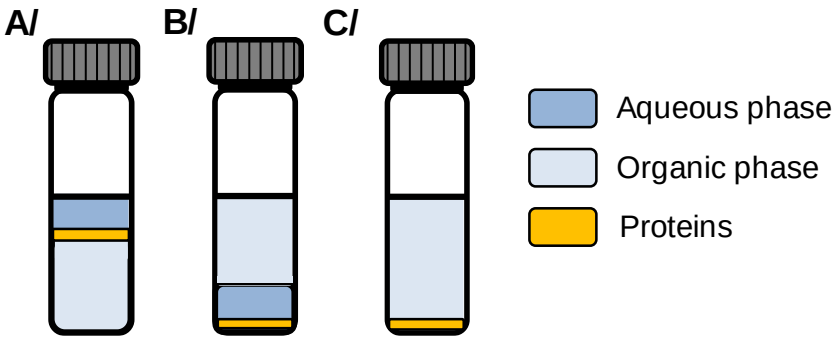

Figure 2 Examples of liquid-liquid extraction using Folch approach (**A/**), MTBE or BUME approach (**B/**), and one-phase extraction (**C/**).

2.2 MS-based methods of lipidomic analysis

Mass spectrometry-based methods (MS-based methods) belong to the main method approaches in the lipidomic research. There are three strategies: chromatography coupled to MS, direct infusion MS method (DI; shotgun MS), and desorption ionization techniques usually used for mass spectrometry imaging (MSI). Every strategy is further classified based on the type of mass spectrometers (e.g., low and high resolution) and/or type of chromatography (lipid species separation and lipid class separation), which was used [17].

The lipid class separation is achieved by hydrophilic interaction chromatography (HILIC), normal-phase (NP) liquid chromatography, and supercritical fluid chromatography (SFC). The lipid species separation can be achieved using silver-ion chromatography, chiral chromatography, or reversed phase liquid chromatography (RP-(U)HPLC), which is the most common and widespread chromatographic mode for lipid species separation [5, 18-20].

Acquiring of MS data usually depends on the concentration of lipids in biological samples. Selection reaction monitoring (SRM) is a typical scan for targeted measurements of oxylipins or other low abundant lipid mediators [16, 21]. Full MS1 or MS/MS scanning are usually used for lipid classes, such as glycerolipids or glycerophospholipids, which are at higher concentrations in biological samples [22].

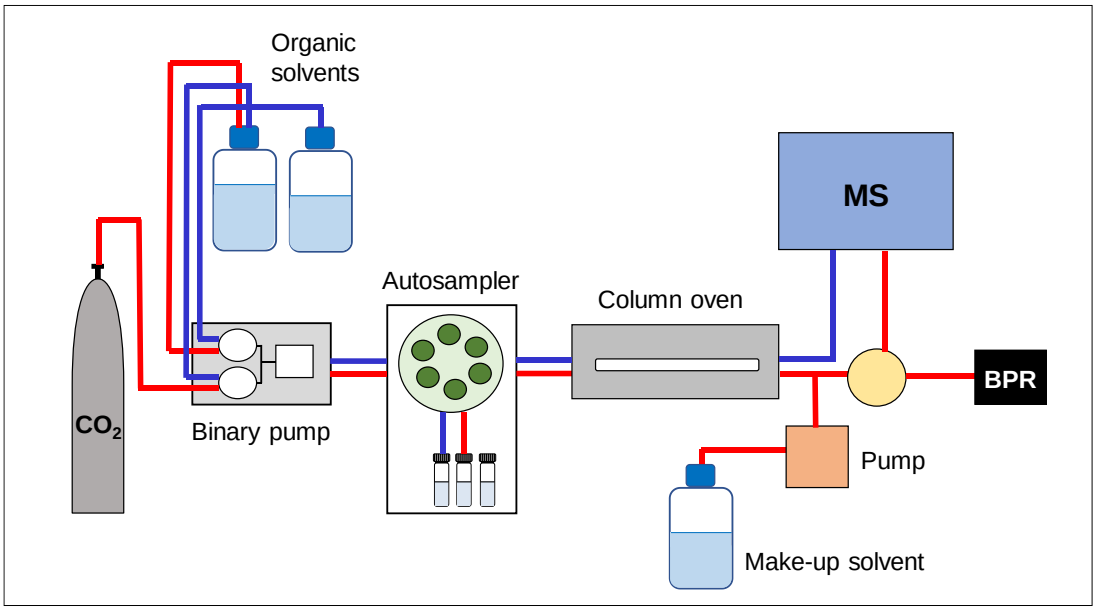

Figure 3 Scheme of HPLC (blue line) and SFC (red line) instrumentation.

3. Quantitative lipidomics

Quantitative approaches are influenced by a selection of MS-based method, what has a connection to a selection of internal standards (IS) and number of IS essential for accurate quantitation. The lipid class separation has

an advantage of the guarantee of the same matrix effects for IS and lipids due to a coelution of IS with endogenous lipids within the same lipid class, but disadvantage is a knowledge only the sum of number of carbons (CN) and number of double bonds (DB). The more detailed information about lipids and their fatty acyl chain structures is given by the lipid species separation, but it is very important to use more IS to cover whole chromatography run [5, 17].

There are two possibilities to express the value of lipid concentration (relative and absolute). Relative concentrations given by a ratio between lipid species express the pattern changes. Absolute concentrations are usually reported as molar concentration and express the total amount of lipids in a testing sample [23].

The crucial part of reliable quantitation is a selection of mixture of IS, which has to be added before extraction and their known concentrations should be close to physiological values. Only this way, the analytes and IS are influenced in the same way during the sample preparation and analysis on basis of recovery rate, matrix effect, or ion suppression. Exogenous IS or isotopically labeled lipid species (e.g., ^{13}C, D) are typically used in the lipidomic analysis. Exogenous IS have shorter, longer fatty acyl chains or odd number of carbons [4, 23].

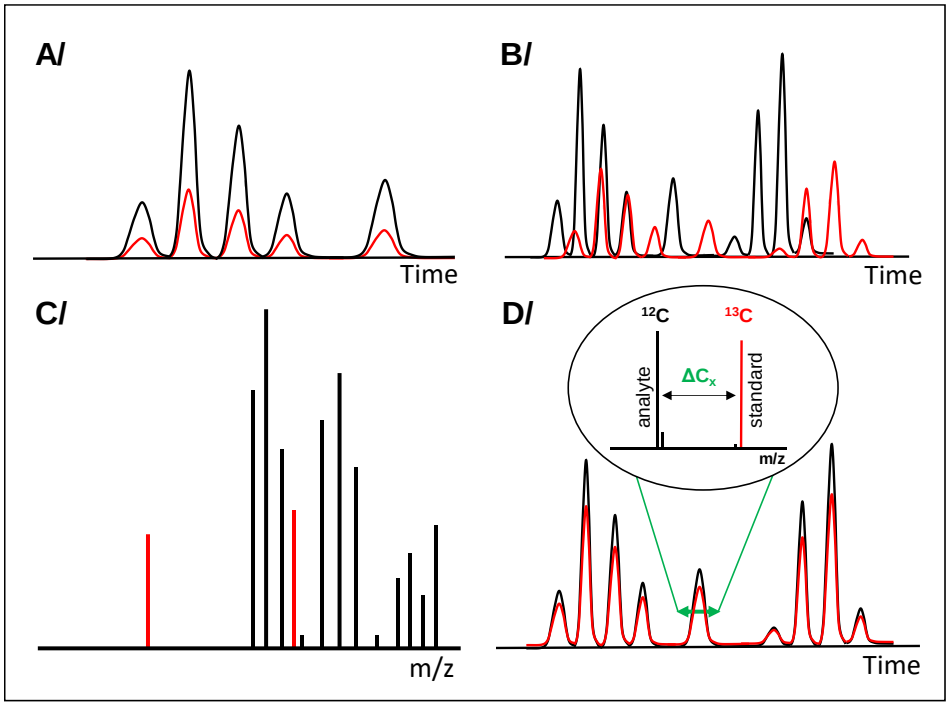

Figure 4 The quantitation approaches. **A/** lipid class separation with at least 1 IS per lipid class; **B/** lipid species separation using more ISs to cover the separation run; **C/** shotgun MS using 2 IS for lipid class (characteristic scan event); **D/** the best quantitation approach using ^{13}C isotopically labeled lipid species as internal standards. Red line – internal standards, black line – endogenous lipids.

3.1 Quality control measurements

To get reliable results about lipid concentrations, it is important to put a special attention to the monitoring and confirming of precision, accuracy, and quality of measurements. This could be achieved by quality control (QC) measurements [24]. QC samples should be of the same nature as studied samples and prepared from the same defined source. Preferentially, it is a pooled sample of representative objects included in the study. Every developed method has to be verified (validated) according to established protocols, to confirm that methods will provide accurate and reproducible results. Relevant guidelines for bioanalytical method validation are provided by the Food and Drug Administration (FDA) [25] or the European Medical Agency (EMA) [26].

The present status in the field of the dissertation topic

The aims of the Ph.D. thesis are to introduce the possibilities of high-throughput lipidomic analysis based on chromatography coupled to mass spectrometry (lipid species separation and lipid class separation) and application of developed methods to the measurements of clinical studies.

Nowadays, there are several methodologies for the analysis of lipids with still some observation of inconsistence of reported data, which can be influenced by wrong annotation of lipids species [27], misidentifications, method error, and diversity of lipid analysis strategies with improper selection of IS [2]. These facts can show us that the field of lipidomics is still challenging and general guidelines including approaches from sample preparation to the data processing are urgently needed.

Goals of the dissertation

A. Overview of mass spectrometric approaches for lipidomic quantitation

- Summary of available MS-based methods of lipidomic analysis and quantitation approaches together with description of the most common biological samples and sample preparation protocols
- Clinical application of lipidomic analysis to cancer research (oncolipidomics)

B. Lipid species separation

- Determination of oxylipins using targeted RP-UHPLC/MS method
- Optimization of SRM method, validation, and application of developed method to clinical human plasma samples of healthy volunteers and breast cancer patients

C. Lipid class separation I

- Determination of glycerolipids (MG, DG, and TG), CE, Cer, SM, LPC, and PC using HILIC-UHPLC/MS and UHPSFC/MS approaches
- Optimization of lipid extraction and validation of HILIC-UHPLC/MS and UHPSFC/MS methods
- Application of developed methods to biological samples (human plasma, human serum, and exosomes) with recommendation of QC measurements
- Comparison of HILIC-UHPLC/MS and UHPSFC/MS methods based on validation parameters and determined lipid concentrations

D. Lipid class separation II (partial contribution)

- Determination of GSL using HILIC-UHPLC/MS and application to measurements of the biological samples

Materials and methods

The whole range of used materials and methods with detailed parameters is described in my published papers related to the topic of this Ph.D. thesis. Therefore, only a brief introduction is discussed.

A. Lipid species separation of oxylipins

63 oxylipin standards and 14 deuterated standards were purchased from Cayman Chemical (Ann Arbor, MI, USA), which were needed for a development of SRM method and a quantitation, respectively.

A reversed-phase ultrahigh-performance liquid chromatography (RP-UHPLC) was used for the separation of oxylipins, which was performed on C18 stationary phase and a mixture of water/acetonitrile/isopropanol with addition of acetic acid as a mobile phase. RP-UHPLC was coupled to the triple quadrupole mass spectrometry using SRM scans.

Oxylipins were determined in human plasma samples. As a sample preparation, a solid-phase extraction was used. First, a protein precipitation of human plasma was performed using acetonitrile, followed by a loading of the supernatant into a catridge with prior cleaning (methanol) and equilibration (water), and then an elution using methanol. C18 columns were used for the solid-phase extraction to enrich a level of oxylipins in extracts.

Human plasma samples of healthy volunteers and breast cancer patients were obtained from the Department of Surgery, Atlas Hospital Zlín in the Czech Republic based on the institutional ethical agreement and signed information consent.

B. Lipid class separation I.

Lipid class separation was used for the analysis of lipids, such as MG, DG, TG, CE, Cer, SM, PC, and LPC. All needed standards and internal standards (deuterated and exogenous) were purchased from Avanti Polar Lipids (Alabaster, AL, USA), Merck (Darmstadt, Germany), or Nu-Chek (Elysian, MN, USA).

A hydrophilic interaction ultrahigh-performance liquid chromatography (HILIC-UHPLC) and an ultrahigh-performance supercritical fluid chromatography (UHPSFC) coupled to a quadrupole – time of flight mass spectrometry (QTOF) were used for determination of mentioned lipids. Both chromatography modes were performed on a silica stationary phases and a mixture of water/acetonitrile/ammonium acetate as a mobile phase in case of HILIC-UHPLC/MS and a supercritical carbon dioxide with methanol/ammonium acetate as a modifier in case of UHPSFC/MS method.

Materials analyzed in this study were biological samples, such as human plasma, human serum, and NIST SRM 1950 Reference material human plasma. The final liquid-liquid extraction approach according to Folch procedures utilizing a mixture of chloroform and methanol was used for sample preparation.

Human plasma and human serum samples were obtained from University Hospital Olomouc in Czech Republic and University of Pardubice based on the institutional ethical agreement and signed information consent.

B. Lipid class separation II.

Glycosphingolipids were purchased from Avanti Polar Lipids (Alabaster, AL, USA).

For the analysis, hydrophilic interaction ultrahigh-performance liquid chromatography (HILIC-UHPLC) coupled to linear ion trap mass spectrometer was used. Separation was performed on a silica stationary phase with mixture of acetonitrile/water/ammonium acetate/acetic acid as a mobile phase.

Human plasma and human serum samples were obtained from University of Pardubice based on the institutional ethical agreement and signed information consent.

Results

A. Overview of mass spectrometric approaches for lipidomic quantitation

The whole overview of possible lipidomics approaches are described with discussion about the quality control measurements, the method validation significant for the reliable quantitation, the lipid classification and nomenclature, the description of the most common biological materials together with the possibilities of the sample preparation protocols and other spectroscopic methods applicable in lipidomic analysis. The main part is focus on MS-based methods described with their realization and quantitation approaches, principles, and data processing requirements.

In recent years, the lipidomic analysis is connected to the cancer research. Therefore, a special attention was given to the evaluation of dysregulated lipid species in various cancers in comparison to healthy state based on published data with the information of analytical method, used IS, and number and sample type of measured subjects.

Reference: WOLRAB Denise, JIRÁSKO Robert, CHOCHOLOUŠKOVÁ Michaela, PETERKA Ondřej, HOLČAPEK Michal. Oncolipidomics: Mass Spectrometric Quantitation of Lipids in Cancer Research. *TrAC: Trends in Analytical Chemistry*, 2019, 120, 115480.

B. Lipid species separation

The best way for the separation of oxylipins is a lipid species separation. It has been developed and optimized method using reversed-phase ultrahigh performance liquid chromatography on C18 column (150 x 2.1 mm, 1.7 μm) with 12 min run time with SRM scanning detection using triple quandrupole mass spectrometer. SRM scans were optimized for 63 oxylipin standards to get the higher sensitivity. Oxylipins have a very low concentration in human plasma samples, therefore, soli-phase extraction was used for sample preparation. Finally, the method was validated and applied to the small cohort of samples of 40 female human plasma samples of breast cancer patients (20) and healthy controls (20) to find possible differences between two mentioned groups. There were observed regulations of some oxylipins in healthy volunteers and breast cancer patients (mainly 13-HODE, 9-HODE, 13-HOTrE, 9-HOTrE, and 12-HHTrE), but the real potential of analysis of oxylipins for cancer screening should be confirmed in larger cohort of samples.

In total, 36 oxylipins were detected in human plasma samples and 21 were quantified using 14 deuterated standards. Determined concentrations were compared with in agreement with the literature values.

Reference: CHOCHOLOUŠKOVÁ Michaela, JIRÁSKO Robert, VRÁNA David, GATĚK Jiří, MELICHAR Bohuslav, HOLČAPEK Michal. Reversed-phase UHPLC/ESI-MS determination of oxylipins in human plasma: case study of female breast cancer. *Analytical and Bioanalytical Chemistry*, 2019, 411, 1239-1251.

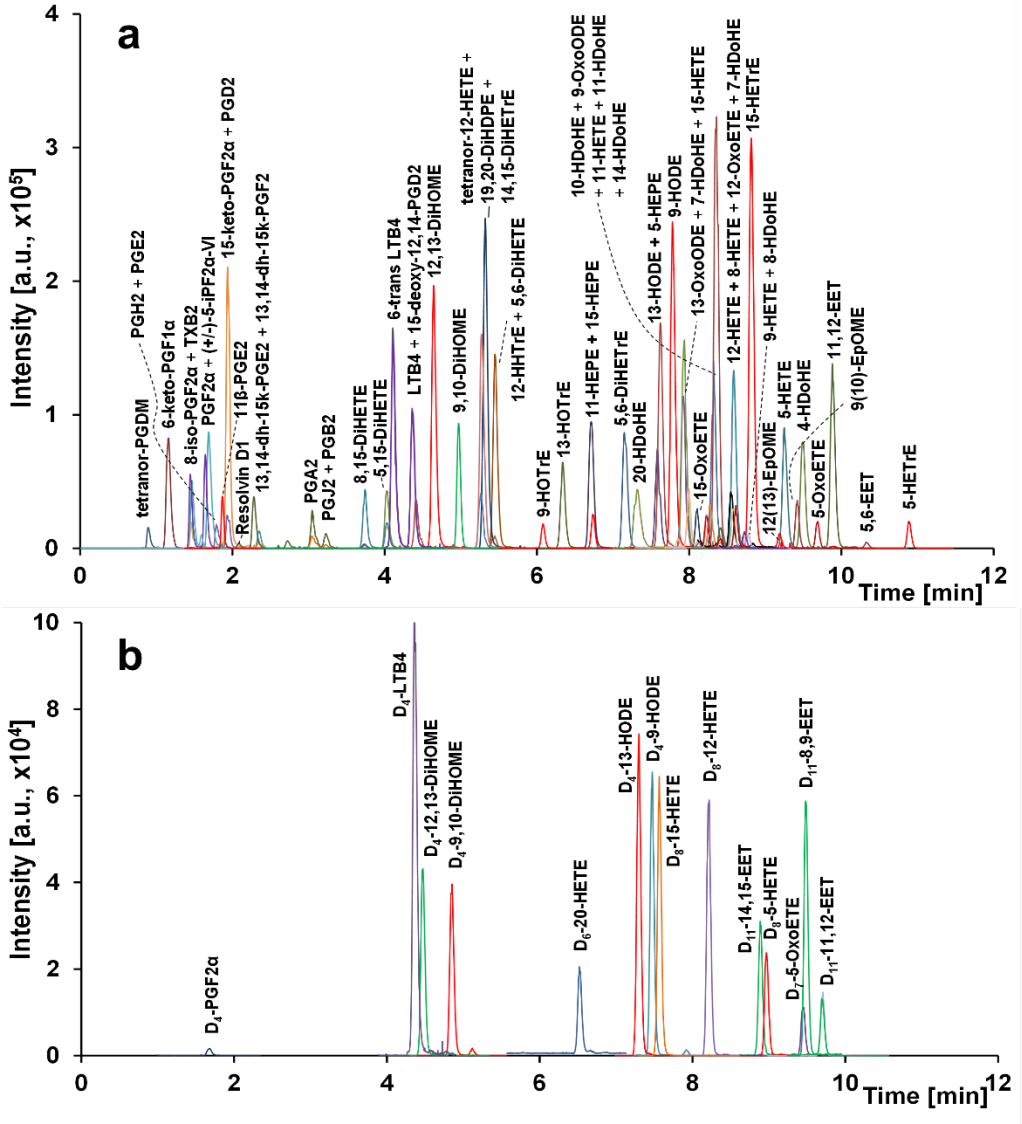

Figure 5 Extracted ion chromatograms in RP-UHPLC/ESI-MS analysis of **a** mixture of 63 oxylipin standards and **b** mixture of 14 deuterated oxylipin internal standards using negative-ion mode and SRM scans [16].

C. Lipid class separation I

HILIC-UHPLC/MS or UHPSFC/MS methods were used for lipid class separation of lipids, such as such as glycerolipids (MG, DG, and TG), cholesteryl-esters (CE), ceramides (Cer), sphingomyelines (SM), lysophosphatidylcholines (LPC), and phosphatidylcholines (PC). These lipids are more abundant in biological samples in comparison to oxylipins, therefore liquid-liquid extraction was used for a sample preparation. Modified Folch approach was evaluated as the best extraction protocol from in total six tried sample preparation protocols. Afterwards, both methods were fully validated using human plasma, human serum, and NIST SRM 1950 human plasma with the investigation of differences between two types of body fluids, differences between two methods, and differences between in-house IS mixture and commercial IS mixture used for the quantitation.

Validation results confirm the applicability of HILIC-UHPLC/MS method as well as UHPSFC/MS method. Big advantages of UHPSFC/MS method is a separation of non-polar and polar lipid classes, shorter separation run, and slightly high robustness.

Developed and validated HILIC-UHPLC/MS and UHPSFC/MS methods were applied to real samples of body fluids of healthy volunteers. The study was focused on effects of blood collection tubes (heparin plasma, EDTA plasma, and serum), one year stability of lipid plasma profile, and the sample origin (two different cohorts) to find possible differences in lipid concentrations obtained from both lipid class separation strategies. No significant differences in lipid profiles were observed on basis of different collection tubes and different period time of blood collection. Lipid concentrations in serum are about 20% higher in comparison to plasma.

Both methods were applied to the measurements of exosomes and plasma sample needed for isolation of exosomes to find differences between these two biological matrices. In total, 244 lipids in exosomes and 191 lipids in plasma within 10 lipid classes were determined. The highest differences between exosomes and plasma were observed for TG, DG, PC, and LPC. Similar lipid profiles were observed for lipid classes, such as SM, PI, and sulfatides.

The special importance was given to the evaluation of a robustness of developed methods. Therefore, HILIC-UHPLC and UHPSFC were connected to two QTOF mass spectrometers to find out the possible differences on basis of measurements of the same sets of samples of healthy volunteers and kidney cancer patients. The results have shown some differences in lipid concentrations, but these variations can be normalized using the NIST Standard Reference Material 1950 human plasma to obtained more consistent results.

References: WOLRAB Denise, CHOCHOLOUŠKOVÁ Michaela, JIRÁSKO Robert, PETERKA Ondřej, HOLČAPEK Michal. 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, 2375-2388.

PETERKA Ondřej, JIRÁSKO Robert, CHOCHOLOUŠKOVÁ Michaela, KUCHAŘ Ladislav, WOLRAB Denise, HÁJEK Roman, VRÁNA David, STROUHAL Ondřej, MELICHAR Bohuslav, HOLČAPEK Michal. Lipidomic characterization of exosomes isolated from human plasma using various mass spectrometric techniques. *Biochimica et Biophysica Acta – Molecular and Cell Biology of Lipids*, 2020, 1865, 158634.

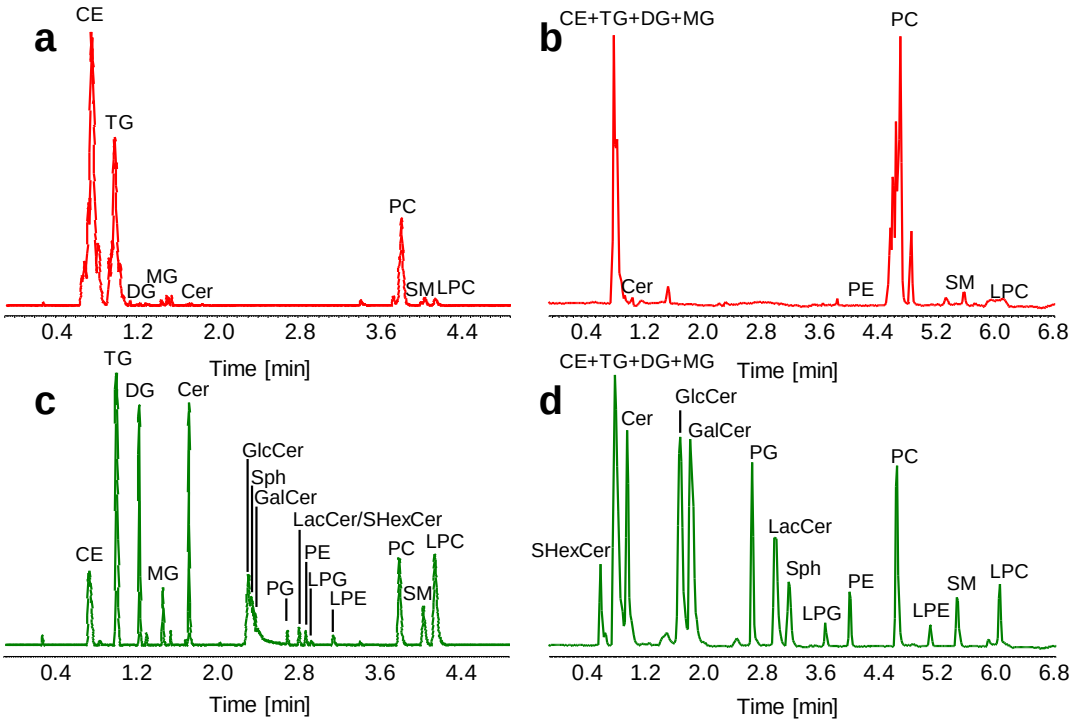

Figure 6 Chromatograms of pooled human plasma spiked with the medium level concentration of IS: **a** UHPSFC/MS and **b** HILIC-UHPLC/MS, and of the system suitability standard: **c** UHPSFC/MS and **d** HILIC-UHPLC/MS

D. Lipid class separation II

HILIC-UHPLC/MS is useful for analysis of glycosphingolipids (GSL) including gangliosides. Their quantitation in biological samples is also challenging due to the structural diversity and their low abundances in biological samples, such as body fluids. The sample preparation using SPE is essential to enrich the concentration of GSL in biological samples. For analyses of GSL, HILIC-UHPLC/MS method was used with the strong emphasis on their structural characterization using MS/MS experiments. The developed method was applied to real samples of human plasma, where 154 GSL within 7 lipid subclasses and 77 phospholipids were reported.

Conclusions

We have shown a lipidomic analysis using MS coupled to chromatography. The Ph.D. thesis is focus on two quantitation strategies for the determination of lipids in biological samples: lipid species separation achieved by RP-UHPLC/MS and lipid class separation achieved by HILIC-UHPLC/MS and UHPSFC/MS.

Lipid species separation approach coupled to tandem mass spectrometry (RP-UHPLC/MS) was applied to the analysis of oxylipin isomers, which is the best way for their comprehensive determination.

Lipid class separation approach coupled to mass spectrometry (HILIC-UHPLC/MS and UHPSFC/MS) was applied to the analysis of lipids, such as glycerolipids (MG, DG, and TG), CE, Cer, SM, LPC, and PC. Developed and fully validated methods were applied to the measurements of the set of clinical samples with the goal to compare UHPSFC/MS method with well-established HILIC-UHPLC/MS method and compare determined lipid concentrations with literature values.

HILIC-UHPLC/MS method is also useful for the determination of glycosphingolipids (GSL) in biological samples. With my partial contribution, the method was developed with the main focus on the structural characterization of GSL in human plasma samples.

List of references

[1] WENK R.M. The emerging field of lipidomics. *Nat. Rev. Drug Discov.* 2005, 4, 594-610.

[2] LIEBISCH G. et al. Lipidomics need more standardization. *Nat. Metab.* 2019, 1, 745–747.

[3] Lipid Maps, Accessed June 20, 2020 at https://www.lipidmaps.org/.

[4] BURLA B. et al. MS-based lipidomics of human blood plasma: a community-initiated position paper to develop accepted quidelines. *J. Lipid Res.*, 2018, 59, 2001-2017.

[5] WOLRAB D. et al. Oncolipidomics: Mass spectrometric quantitation of lipids in cancer research. *TrAC* 2019, 120 115480.

[6] CÍFKOVÁ E. et al. Correlation of lipidomic composition of cell lines and tissues of breat cancer patients using hydrophilic interaction chromatography /electrospray ionization mass spectrometry and multivariate data analysis. *Rapid. Commun. Mass Spectrom.* 2017, 31, 253-263.

[7] CÍFKOVÁ E. et al. Determination of lipidomic differences between human breast cancer and surrounding normal tissue using HILIC-HPLC/ESI-MS and multivariate data analysis. *Anal. Bioanal. Chem.* 2015, 407, 991-1002.

[8] CÍFKOVÁ E. et al. Lipidomic differentiation between human kidney tumors and surrounding normal tissues using HILIC-HPLC/ESI-MS and multivariate data analysis. *J. Chromatogr. B*, 2015, 1000, 14-21.

[9] GHOSH A., NISHTALA K. Biofluid lipidome: a source for potential diagnostic biomarkers. *Clin. Transl. Med.*, 2017, 6, 6-22.

[10] POCSFALVI G. et al. Mass spectrometry of extracellular vesicles. *Mass Spectrom. Rev.*, 2016, 35, 3-21.

[11] PETERKA O. et al. Lipidomic characterization of exosomes isolated from human plasma using various mass spectrometric techniques. *BBA-Mol. Cell Biol L.*, 2020, 1865, 158634.

[12] FOLCH J. et al. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.*1957, 226, 497-509.

[13] BLIGH E.G. et al. A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, 1959, 37, 91-917.

[14] MATYASH V. et al. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. *J. Lipid Res.*, 2008, 49, 1137-1146.

[15] LÖFGREN L. et al. The BUME method: a new rapid and simple chloroform-free method for total lipid extraction of animal tissue. *Sci. Rep.*, 2016, 6, 27688.

[16] CHOCHOLOUŠKOVÁ M. et al. Reversed phase UHPLC/ESI-MS determination of oxylipins in human plasma: a case study of female breast cancer. *Anal. Bioanal. Chem.*, 2019, 411, 1239-1251.

[17] HOLČAPEK M. et al. Lipidomic analysis. *Anal. Chem.*, 2018, 90, 4249-4257.

[18] LÍSA M. et al. Lipidomic analysis of biological samples: comparison of liquid chromatography, supercritical fluid chromatography and direct infusion mass spectrometry methods. *J. Chromatogr. A,* 2017, 1525, 96-108.
[19] ČAJKA T. et al. Comprehensive analysis of lipids in biological systems by liquid chromatography-mass spectrometry. *TrAC,* 2014, 61, 192-206.
[20] LÍSA M. et al. High-throughput and comprehensive lipidomic analysis using ultrahigh-performance supercritical fluid chromatography-mass spectrometry. *Anal. Chem.,* 2015, 87, 7187-7195.
[21] WANG Y. et al. Comprehensive ultra-performance liquid chromatographic separation and mass spectrometric analysis of eicosanoids metabolites in human samples. *J. Chromatogr. A,* 2017, 1359, 60–69.
[22] LÍSA M. et al. Comparison of Liquid Chromatography, Supercritical Fluid Chromatography and Direct-Infusion Mass Spectrometry Methods for Lipidomic Analysis. *J. Chromatogr. A,* 2017, 1525, 96-108.
[23] WANG M. et al. Selection of internal standards for accurate quantification of complex lipid species in biological extracts by electrospray ionization mass spectrometry - what, how and why? *Mass Spectrom. Rev.,* 2017, 36, 693-714.
[24] BROADHURST D. et al. Guidelines and considerations for the use of system suitability and quality control samples in mass spectrometry assays applied in untargeted clinical metabolomic studies. *Metabolomic,* 2018, 13, 50.
[25] US Department of Health and Human Services (FDA). 2018. Bioanalytical method validation: Guidance for industry. Accessed July 20, 2020, at https://www.fda.gov/files/drugs/published/Bioanalytical-Method-Validation-Guidance-for-Industry.pdf
[26] European Medicines Agency (EMA). 2015. Guideline on bioanalytical method validation. Accessed July 20, 2020, at https://www.ema.europa.eu/en/documents/scientific-guideline/guideline-bioanalytical-method-validation_en.pdf
[27] BOWDEN A.J. et al. Harmonizing lipidomics: NIST interlaboratory comparison exercise for lipidomics using SRM 1950–Metabolites in Frozen Human Plasma. *J. Lipid Res.,* 2017, 58, 2275-2288.

List of Published Works

WOLRAB Denise, JIRÁSKO Robert, CHOCHOLOUŠKOVÁ Michaela, PETERKA Ondřej, HOLČAPEK Michal. Oncolipidomics: Mass Spectrometric Quantitation of Lipids in Cancer Research. *TrAC: Trends in Analytical Chemistry*, 2019, 120, 115480.

CHOCHOLOUŠKOVÁ Michaela, JIRÁSKO Robert, VRÁNA David, GATĚK Jiří, MELICHAR Bohuslav, HOLČAPEK Michal. Reversed-phase UHPLC/ESI-MS determination of oxylipins in human plasma: case study of female breast cancer. *Analytical and Bioanalytical Chemistry*, 2019, 411, 1239-1251.

WOLRAB Denise, CHOCHOLOUŠKOVÁ Michaela, JIRÁSKO Robert, PETERKA Ondřej, HOLČAPEK Michal. 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, 2375-2388.

PETERKA Ondřej, JIRÁSKO Robert, CHOCHOLOUŠKOVÁ Michaela, KUCHAŘ Ladislav, WOLRAB Denise, HÁJEK Roman, VRÁNA David, STROUHAL Ondřej, MELICHAR Bohuslav, HOLČAPEK Michal. Lipidomic characterization of exosomes isolated from human plasma using various mass spectrometric techniques. *Biochimica et Biophysica Acta – Molecular and Cell Biology of Lipids*, 2020, 1865, 158634.

Published work not related to my Ph.D. thesis

CHOCHOLOUŠKOVÁ Michaela, KOMENDOVA Martina, URBAN Jiří. Retention of small molecules on polymethacrylate monolithic capillary columns. *Journal of Chromatography A*, 2017, 1488, 85-92.