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

Ph.D. thesis

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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.

Keywords:

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

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.

Klíčová slova:

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

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Abbreviations

2D-LC	two-dimensional liquid chromatography
AA	arachidonic acid
acetyl-CoA	acetyl-coenzyme A
acyl-CoA	acyl-coenzyme A
Ag-HPLC	silver-ion high-performance liquid chromatography
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
BMI	body mass index
BPR	back pressure regulator
BQC	batch quality control
BUME	butanol/methanol
CN	carbon number
CO_2	carbon dioxide
COX	cyclooxygenase
CYP450	cytochrome P450
DB	double bond
DG	diacylglycerol
DHA	docosahexanoeic acid
DI	direct infusion
ECN	equivalent carbon number
EMA	European Medical Agency
EPA	eicosapentanoeic acid
ESI	electrospray ionization

FA	fatty acid
FDA	Food and Drug Administration
FT	Fourier transformation
GC	gas chromatography
GL	glycerolipid
GP	glycerophospholipid
GSL	glycosphingolipid
HDL	high-density lipoprotein
HILIC	hydrophilic interaction chromatography
ICR	ion cyclotron resonance
IS	internal standard
LA	linoleic acid
LC	liquid chromatography
LDL	low-density lipoprotein
LnA	linolenic acid
LOX	lipoxygenase
LPC	lysophosphatidylcholine
m/z	mass to charge
MALDI	matrix-assisted laser desorption/ionization
malonyl-CoA	malonyl-coenzyme A
MG	monoacylglycerol
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSI	mass spectrometry imaging

MTBE	methyl *tert*-butyl ether
NARP	non-aqueous reversed phase
NP	normal-phase
OPLS-DA	orthogonal partial least squares discriminant analysis
PA	phosphatidic acid
P_c	critical pressure
PC	phosphatidylcholine
PCA	principal component analysis
PE	phosphatidylethnanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PK	polyketide
PLA2	phospholipase A2 enzyme
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
Q	quadrupole
QC	quality control
QqQ	triple quadrupole
(Q)-TOF	(quadrupole) - time of flight analyzer
QTRAP	quadrupole - linear ion trap analyzer
RP	reversed-phase
SL	saccharolipid
SM	sphingomyelin
SP	sphingolipid

SPE	solid-phase extraction
SRM	selective ion monitoring
ST	sterol lipid
T_c	critical temperature
TG	triacylglycerol
TQC	total quality control
(U)HPLC	(ultra)high-performance liquid chromatography
(U)HPSFC	(ultra)high-performance supercritical fluid chromatography
VIP	variable importance in projection

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1 Introduction

1.1 Lipidomics

Lipids are highly structural diverse biomolecules, differing in their various combinations of fatty acyls on backbone structures (e.g., glycerol or sphingoid base). The study of lipids (the lipidome) in cells or organism and their explorative study of their structures, functions, and interactions with proteins, metabolites, or other lipids is called lipidomics [1]. The lipidomics is a fast-developing science, however, the term "lipid" still does not have a satisfactory general definition. Christie describes the lipid definition as follows: "Lipids are fatty acids and their derivatives, and substances related biosynthetically or functionally to these compounds" [2]. The Lipid MAPS consortium has established their own definition of lipids as "hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters (fatty acids, polyketides, etc.) and/or by carbocation-based condensations of isoprene units (prenols, sterols, etc.)" [3]. Another definition (broadly described) is that lipids are naturally occurring compounds soluble in organic solvents and insoluble in water [4]. The term lipidomics was first used in 2002 (according to Web of Science) and since then the number of published papers is increasing every year (**Figure 1**).

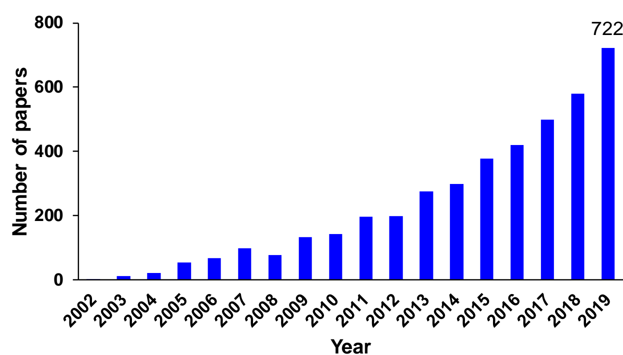


Figure 1 Number of published papers for the keyword "Lipidomics" according to Web of Science searched from May 26 2020.

For a long time, it was expected that lipids have only functions in the storage of energy and building blocks of cell membranes. Recently, it has been discovered that lipids have other important functions. As far back as in 1929, George and Mildred Burr claimed the importance of linoleic acid as an essential dietary constituent. In 1964, Bergström, Samuelsson, and others came with an assertion of essential arachidonic acid as a biosynthetic precursor of prostaglandins with their effects on inflammation and other disease states. In 1979, the first biologically active phospholipid, platelet-activating factor [5], was discovered, and the scientific world has begun to realize an importance of lipids in human bodies (**Figure 2**) [6]. At present time, only analysis of lipids, such as total cholesterol, cholesterol LDL (low-density lipoprotein), cholesterol HDL (high-density lipoprotein), total triacylglycerols, free fatty acids,

bile acids, and lipid-based hormones (progesterone, estradiol, testosterone, and cortisol) are routinely analyzed in common clinical practice [7, 8].

Organelle´s and cell´s membranes have characteristic lipid compositions, where lipids do not behave as single compounds, but mostly as a collective of molecules with importance of comprehensive and quantitative analysis of lipids to understand their role in organism. The lipids are relatively small hydrophobic and signaling molecules, which may reflect the metabolic state of the organism and diseases, such as cancer, atherosclerosis, Alzheimer´s disease, obesity, heart diseases, etc. [9]. The analysis of lipids was challenging due to the complexity of naturally occurring lipids and their concentrations over several orders of magnitude. The commercialization of sophisticated/advanced instruments combining chromatography and mass spectrometry facilities the analysis of lipids and led to the great interest of lipid research.

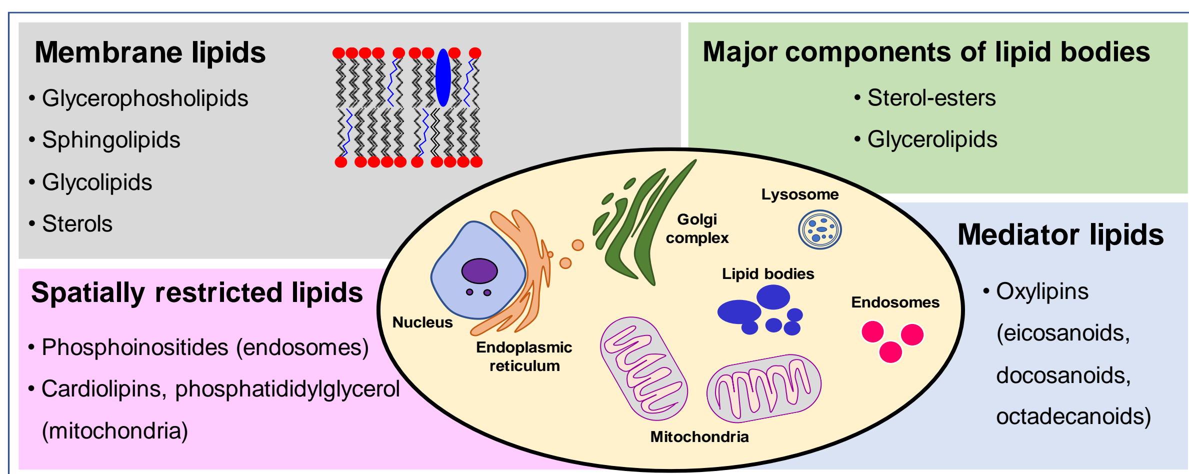


Figure 2 Importance of lipid classes in human body.

Generally, lipids can be divided into two groups – "simple" lipids yielding at most two products after the hydrolysis (fatty acids, acylglycerols, etc.) and "complex" lipids yielding three or more products after the hydrolysis (glycerophosholipids, etc.). 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) [4].

Fatty acyl groups are major building blocks of complex lipids. They are formed by chain elongation of an acetyl-CoA primer with malonyl-CoA (or methylmalonyl-CoA) groups, where palmitic acid is the final product of fatty acyl biosynthesis. This acid may be further prolonged and/or desaturated to other fatty acids. Many variants in their structures including different number of carbons, different numbers and positions of double bonds (even triple bonds), cyclic formations, substitutions with heteroatoms, and others [4, 10] have been reported. Only two

essential fatty acids, which cannot be generated in the human body, are linolenic (LnA) and linoleic acid (LA), important for the biosynthesis of polyunsaturated fatty acids (PUFA) further used as precursors of oxylipins having significant biological activities [11]. Arachidonic acid (AA), eicosapentanoeic acid (EPA), and docosahexanoeic acid (DHA) are examples of acids belonging to PUFA [11-15].

Oxylipins are bioactive lipids connected to ongoing inflammation in the human body. Their important functions are apoptosis, cell proliferation, or immune reactions [16]. Eicosanoids are the best known oxylipin´s group generated from 20-carbon PUFA (AA, EPA etc.). Other oxylipin´s groups are docosanoids derived from 22-carbons PUFA (EPA, DHA, and docosatetranoeic acid) and octadecanoids derived from 18-carbons PUFA (LA, LnA) [11]. Oxylipins can be generated either by the enzymatic pathway or non-enzymatic pathway, but first the PUFA have to be released from glycerophospholipid structure (usually from *sn-2* position) by the phospholipase A2 enzyme (PLA2). For the enzymatic pathway of oxylipins, three important enzymes are involved: cyclooxygenase (COX), lipoxygenase (LOX), and cytochrome P450 (CYP450) [12-14, 17-19].

The basis of glycerolipids is the glycerol substituted with fatty acids via ester bonds. GL are divided into mono-, di-, and tri-acylglycerols (MG, DG, and TG, respectively) according to the number of esterific fatty acids. The presence of one or more sugar residues attached to glycerol via a glycosidic bond characterized another subclass of GL called glyceroglycolipids [4, 20].

Glycerophospholipids are key components of cell membranes (cytoplasmatic membrane of eukaryotic cell) [21]. GP are subdivided into distinct classes according to the polar headgroup at the *sn-3* position of the glycerol backbone. Examples are phosphatidylcholines (PC), phosphatidylethnanolamines (PE), phosphatidic acids (PA), phosphatidylglycerols (PG), phosphatidylinositoles (PI), and phosphatidylserines (PS). In case of absence of the fatty acyl in *sn-1* or *sn-2* position of the glycerol backbone, lipids are called lysoglycerophospholipids [4].

Sphingolipids share a common structure feature – a sphingoid base backbone synthesized de novo from serine and a long-chain fatty acyl-CoA and then further converted into ceramides, phosphosphingolipids, or glycosphingolipids (GSL). The major sphingoid base (in mammals) is a sphingosine and the most common sphingolipid species are sphingomyelins (SM) [4, 22]. GLS are divided according to the nature of a saccharide head group into other groups, such as monoglycosylceramides (cerebrosides) and oligoglycosylceramides

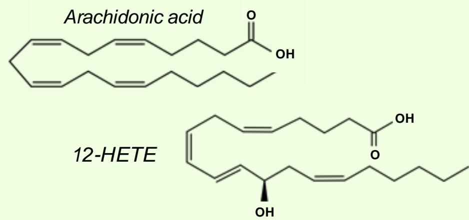
(globosides) belonging to the neutral GSL and sialosylglycosylceramides (gangliosides) and sulfoglycosylceramides (sulfatides) belonging to the acidic GSL [23, 24].

Sterol lipids, the most common example is cholesterol and its derivatives, are polycyclic molecules of unsaturated alcohols and aliphatic chains. Prenol lipids share one or more isoprenoid unites. Polyketides are very complex lipids including cyclic parts, and their chains can be further modified by chemical reactions, such as methylation, oxidation, or glycosylation. Saccharolipids are characterized by sugar units directly bonded to fatty acids instead of glycerol backbone as for glycerolipids or glycerophosholipids [4].

To better understand the lipidomic data within the research community, it is necessary to have a proper/unified annotation and structure information of these mentioned complex compounds and a solution can be using the shorthand notation explained [25] by an example of phosphatidylcholine (PC) 34:2. The abbreviation PC describes the phosphatidylcholine lipid class, then, numbers 34 and 2 reflect the number of carbon atoms (CN) and the number of double bonds (DB), respectively. Detailed fatty acids linked to the glycerol backbone have to be experimentally proven and described by the following rules: underline (_) is used when the *sn*-position of fatty acids in not known (PC 18:1_16:1) and slash (/) is used when *sn*-position is already proven (PC 18:1/16:1).

The diversity of lipids with most common lipid species is shown in **Figure 3**. Nowadays, more than 40,000 lipid species are known and described by their structures [4]. Although there are several methodologies for the analysis of lipids in recent years, still, inconsistency is observed in published data [26]. The irreproducibility is mainly caused by improper annotation of lipid species, misidentifications, software errors, or diversity of lipid analysis strategies [9]. These facts prove that lipidomic research is quite challenging, and general guidelines for lipidomic workflows are needed for the entire process, from the preanalytics to sample preparation and measurements as well as data analysis and reporting.

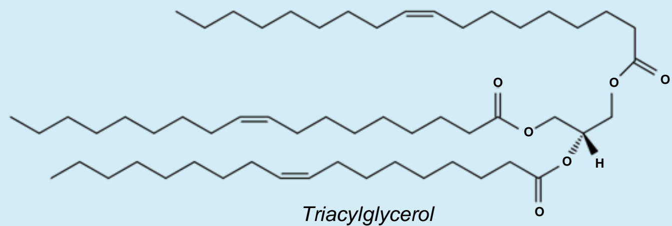
1/ Fatty acyls



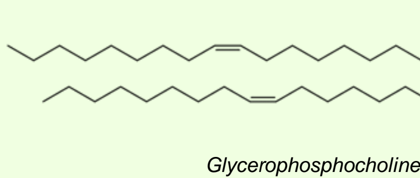
- Fatty acids and conjugates (straight chain, branched, unsaturated, oxo, epoxy, amino, cyano, nitro, carbocyclic fatty acids etc.)
- Octadecanoids, docosanoids, eicosanoids
- Fatty alcohols, fatty aldehydes, fatty esters
- Fatty amides, fatty ethers, hydrocarbons etc.

2/ Glycerolipids

- Monoacylglycerols
- Diacylglycerols
- Triacylglycerols
- Glycosylmonoacylglycerols
- Glycosyldiacylglycerols



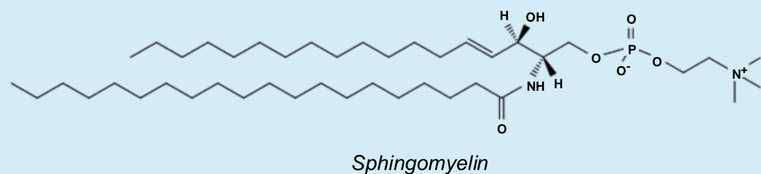
3/ Glycerophospholipids



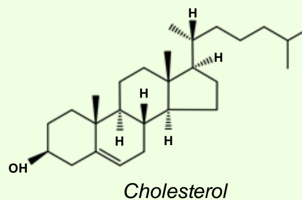
- Glycerophosphocholines
- Glycerophosphoethanolamines
- Glycerophosphoserines
- Glycerophosphoglycerols
- Glycerophosphoinositoles
- Glycerophosphates

4/ Sphingolipids

- Sphingoid bases
- Ceramides
- Phosphosphingolipids
- Glycosphingolipids

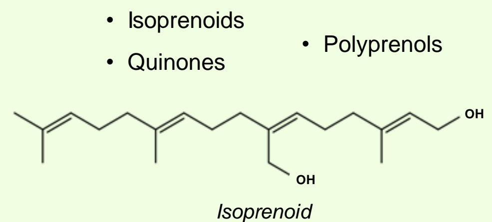


5/ Sterol lipids and 6/ prenol lipids

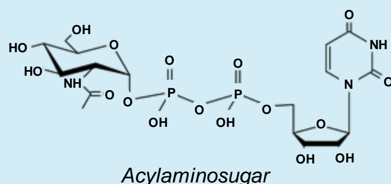


- Sterols
- Steroids
- Secosteroid
- Bile acids

- Isoprenoids
- Quinones
- Polyprenols



7/ Saccharolipids and 8/ polyketides



- Acylaminosugars
- Acylaminosugar glycans
- Acyltrehaloses

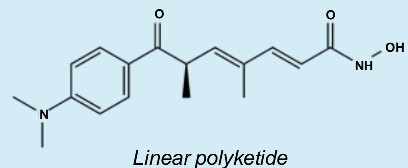


Figure 3 Lipid classification according to Lipid MAPS.

1.2 Lipidomic analysis

For the lipidomic analysis, several steps are important to be considered, such as the study design, the type and description of samples together with clinical data, collection of samples, and their storage (called as preanalytics). This part is crucial for final evaluation and study output as well as quantitative results, besides sample preparation (extraction), sample and quality control (QC) measurements, data processing, and statistical evaluation [8].

It is important to know expectations and hypotheses of the research before lipidomic analysis, in order to define the choice of samples, the size of the sample set, the type of samples, or different groups of samples. Biological samples needed for lipidomic research are usually obtained from third parties, such as clinics or biobanks, and every volunteer had to sign an ethical agreement to use personal and clinical data. The minimum necessary personal and clinical information of volunteers is age, gender, body mass index (BMI), but of course the knowledge of other parameters, such as fasting status of volunteers, medications (especially these influencing lipid metabolism), smoking status, alcohol consumption, diet, diabetic status [8, 27-30], and in case of differentiation of 2 or more groups (for example healthy volunteers and patient with any disease, such as cancer) to have detailed information about type and stage of disease are may of interest as well for the evaluation of characteristic lipidomic profiles.

1.2.1 Biological materials

Body fluids, such as plasma or serum, are the most common used biological material for the analysis of human health state. The advantage is that the blood collection is almost non-invasive, easily feasible and therefore popular in clinical practice [8]. Plasma and serum are collected from whole venous blood to the tubes, which may have some additives. Plasma is isolated from anticoagulated blood, where the coagulation is prevented using additives (anticoagulants), such as EDTA, heparin-lithium, or citrate [31-38]. Serum is isolated from coagulated blood and various analytes including lipids can be released from platelets, erythrocytes, and leukocytes either in extracellular vesicles or in soluble forms during the clotting process. This may affect lipid concentrations due to degradation or generation of lipid species, such as oxylipins, lysophospholipids, or sphingosine 1-phosphate (S1P) [32, 39-41]. Therefore, the use of plasma samples is recommended in the lipidomic research, especially EDTA plasma is suggested as the most convenient biological matrice [8].

The blood collection and separation of plasma/serum from blood cells should be done according to established protocols to minimize hemolysis, platelet activation, or hemoconcentration due to venous stasis. The whole process should be performed at constant conditions within a research study to prevent some misinterpretations based on sample treatment artifacts [42, 43]. For example, in case of serum, it is important to keep the blood sample for some time at ambient temperature to ensure the complete clotting [8]. After the isolation of plasma/serum, samples should be immediately frozen at least at -80°C to prevent oxidation or metabolomic processes influencing metabolite concentrations. Some lipid species, such as oxylipins or other oxidized compounds are more prone to the degradation than other lipids, such as TG or glycerophospholipids. Freeze-thaw cycles should be kept at a minimum or at least constant within a study [40, 44-47].

Generally, lipids can be determined in all common biological matrices [7]. There are several papers about the determination of lipids in cell lines, tissues [48-50], urine, cerebrospinal fluid, saliva [51], recently even in extracellular vesicles (exosomes) [52, 53], and in capillary blood, what brings advantages, such as fast sampling (usually finger prick), but disadvantages in the form of contamination with used cosmetics or skin tissue fluid what could be reflected in the robustness of sample preparation [8].

1.2.2 Lipid extraction

Lipid extraction is commonly used for the sample preparation in the lipidomic analysis. The main aim of the sample preparation protocol is to reduce the complexity of the sample matrix, i.e., by removing proteins and metabolites. However, the optimization of the speed, the robustness, and the repeatability of the extraction protocol should be considered. The recovery and repeatability of lipids can be improved by the optimization of each step of sample preparation, such as used solvents, temperature, pH, volumes of biological samples and solvents, time for centrifugation, sonication, vortexing, and used materials, i.e., plastic or glass vials. Generally, it is strongly recommended to validate the sample preparation protocol, which may help to evaluate possible errors in the sample preparation protocol [8].

The most common approach of sample preparation for lipidomic analysis is liquid-liquid extraction, which can differ by used solutions. The most common protocol is based on a mixture of chloroform/methanol (2:1, *v/v*) introduced by Folch [54] or Bligh-Dyer using a mixture of chloroform/methanol at vice versa ratio (1:2, *v/v*) [55], both followed by addition of water. Other commonly used liquid-liquid extractions are the use of methyl *tert*-butyl ether (MTBE)

and methanol mixtures followed by the addition of water [56], or the mixture of butanol-methanol (BUME) followed by the addition of heptan/ethyl acetate mixture and 1% of acetic acid in water [57]. All mentioned protocols are two-phase extraction protocols. The advantage of MTBE and BUME approaches is that the organic phase containing almost all lipids is the upper one, which is more comfortable for the isolation of the organic phase in contrast to chloroform-based approaches, where the organic phase is the lower phase (**Figure 4**). Liquid-liquid extraction approaches can be also modified as for instance by adding different additives (ammonium acetate, ammonium formate, or ammonium carbonate etc.) during sample preparation to improve the extraction recovery [58].

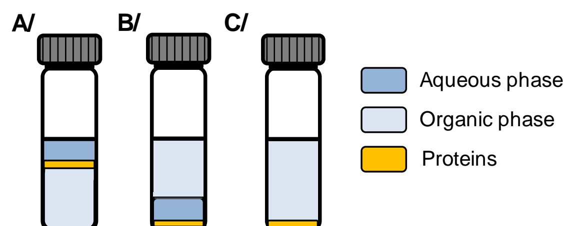


Figure 4 Examples of liquid-liquid extraction using Folch approach (**A/**), MTBE or BUME approach (**B/**), and one-phase extraction (**C/**).

One-phase extractions are used in lipidomic research as well, using one solution like methanol or a mixture of solutions (for example butanol/methanol), triggering protein precipitation. However, the further purification of the extract is missing due to the absence of an aqueous phase, as in case of two-phase extraction [7]. On the other hand, the protein precipitation is a fast and simple method for the analysis of biological samples, which may improve the recovery of polar analytes in comparison to classical two-phase extractions.

If higher selectivity of extraction is needed, then solid-phase extraction (SPE) can be applied using pure silica or octadecylsilica cartridges depending on the nature of investigated lipids. The enrichment and fractionation of lipids is especially suitable for extractions of polar or ionic lipids from aqueous phase as well as for a low abundant lipid species in biological matrices [16].

1.2.3 MS-based methods of lipidomic analysis

The main approaches in the field of lipidomics are MS-based methods, such as MS coupled to chromatography, direct infusion MS method (DI; shotgun MS), and desorption ionization techniques usually used for mass spectrometry imaging (MSI). These lipidomic approaches can be further classified based on the type of mass spectrometers and/or type of chromatography used [59].

1.2.3.1 Direct infusion - MS

The direct infusion approach connected to low-resolution MS [60] or high-resolution MS [61, 62] has the advantage of constant ion suppression effects within lipid classes and lipid species due to the same ionization at the same time. The samples are slowly and directly infused to the ion source using either flow injection or robotic systems, such as TriVersa NanoMate [7]. The principle is based on measurements of specific MS/MS scans, as almost all lipid classes and subclasses are characterized by specific fragments or neutral losses (mainly in the positive ion mode) allowing them to be determined in biological samples (**Table 1**) [59, 63-65]. Precursor ion scans and neutral loss scans are the most common MS/MS approaches for shotgun MS analysis. In the negative ion mode, fragment ions of hydrocarbon chains of lipid molecules are occurring in the spectra allowing to specify lipid species, but for the assignment of *sn*-position of hydrocarbon chains in the glycerol backbone (glycerolipids and glycerophospholipids) more advanced methods are needed. In case of high-resolution MS [66-68], lipid can be identified due to the high mass accuracy, allowing the determination of the elemental composition of lipid species (from precursor ions and product ions) using very narrow Δ*m/z* tolerance settings [62, 69].

Table 1 Product ions and neutral losses of most common lipid species in positive-ion mode.

Lipid class	Product ion	Neutral loss
CE	369	-
Cer	264	-
DG	-	35
MG	-	35
PA, LPA	-	115
PC, LPC, SM	184	-
PE, LPE	-	141
PG, LPG	-	189
PI, LPI	-	277
PS, LPS	-	185
SHexCer	-	98
TG	-	17

1.2.3.2 Chromatography

Two chromatographic approaches can be distinguished for lipid separation, lipid class separation and lipid species separation. Both approaches have advantages and limitations (mainly connected to quantitation). The lipid class separation is achieved by hydrophilic interaction chromatography (HILIC), normal-phase (NP) liquid chromatography and supercritical fluid chromatography (SFC). Reversed phase liquid chromatography (RP-(U)HPLC) is the most used and widespread chromatographic mode for lipid species separation [60, 64, 70]. However, lipid species separation can also achieve with some other chromatographic modes, such as non-aqueous reversed phase chromatography (NARP) [71], silver-ion chromatography (Ag-HPLC) [72] or chiral chromatography [73]. For better

chromatographic resolution and separation speed, sub-2 µm particles columns can be used in so called ultrahigh-performance liquid chromatography (UHPLC) and ultrahigh-performance supercritical fluid chromatography (UHPSFC).

1.2.3.2.1 High-performance liquid chromatography

HPLC belongs to the most powerful, useful, and widely used tool for separation, identification, and quantitation of analyte mixtures soluble in a liquid. HPLC is exceptional due to the broad applicability range, great precision, and commercial wide range of columns, materials, and other equipment.

The beginning of chromatography is ascribed to Mikhail Tswett (in the early 1900s), who was able to separate plant extracts for the first time. A few years later, classical column chromatography was expanded and further developed to other types of chromatography, such as thin-layer chromatography, ion-exchange chromatography, and gel-permeation chromatography. In 1960s, the first HPLC system (column with particles of small diameters and instruments capable to deal with high pressure) was introduced under the leadership of Csaba Horváth and Josef Huber [74].

The chromatography principle is based on interaction of analyte mixtures between mobile phase and stationary phase, where separation mechanism (adsorption or partition) depends on physico-chemical properties of mentioned phases and the analytes [74]. Different chromatographic modes useful for lipidomic analysis are described in more detail in following subchapters.

Reversed phase liquid chromatography (RP-HPLC)

RP-HPLC uses hydrophobic stationary phases, such as C8 or C18 (bonded on silica gel), which are the most common in the lipid research and the mixture of water and organic solvents (acetonitrile, methanol, isopropanol, etc.) with additives (buffer, acids, or bases) as the mobile phase. The mobile phase has a polar character, while the stationary phase is non-polar. Retention is based on hydrophobic interactions between analytes and a stationary phase and increases with decreasing polarity of analytes and decreases with lower percentage of water in the mobile phase [74]. Lipids with longer fatty acyl chains have a stronger hydrophobic interaction with the stationary phase than lipids with shorter fatty acyl

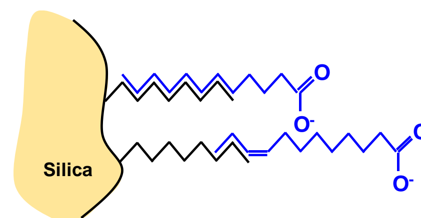


Figure 5 Hydrophobic interactions between lipids and stationary phase.

chains, which results in longer retention times. The presence of double bonds in the fatty acyl chain may diminish the hydrophobic interaction due to sterical hindrance, leading to faster elution. The polar headgroup of lipids also influences the retention behaviour, the more polar the faster the elution. Generally, the retention increases with increasing equivalent carbon number (ECN).

RP-HPLC is especially powerful for the separation of isomers and structural closely related molecules differing in the number or position of double bonds or substituents. The high performance of RP methods to separate structural closely related compounds were recently shown in the separation of 184 oxylipins in 5 min [14], over 400 lipid species within 14 lipid classes in human plasma, urine, and porcine brain samples [75], or the identification and quantification of sphingolipids (SM, Cer, HexCer, Hex_2Cer etc.) in biological samples [76].

Hydrophilic interaction liquid chromatography (HILIC)

HILIC mode uses polar stationary phases and the aqueous-organic mobile phase (at least 2-3% of water in the mobile phase is required, and acetonitrile is the most commonly used organic solvent). The retention behavior is opposite to the RP retention behavior. More polar analytes have higher retention, and water has the higher elution strength. In HILIC mode, less polar and non-polar compounds are eluted in the void volume or close to the void volume. The advantages of HILIC mode are enhanced sensitivity in case of mass spectrometry coupling due to the mobile phase composition, the possibility to use higher flow rates due to the lower mobile phase viscosity, and improved peak shapes of basic analytes [74, 77].

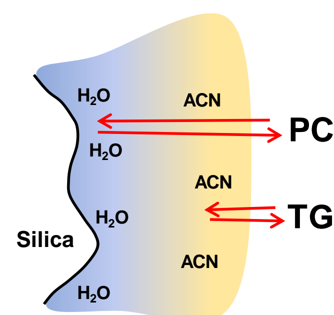


Figure 6 Principle of HILIC separation.

The separation principle is still not completely understood, but it is assumed that a water layer on the surface of the polar stationary phase followed by an aprotic layer are formed. Separation is then achieved depending on the polarity or solubility of the analytes, whereby polar analytes are better soluble in the water rich layer than the aprotic layer. It is assumed that retention is based on partition and adsorption mechanisms, as polar analytes migrate to the water rich layer close to the surface of the stationary phase, facilitating interactions with the selector bonded on the stationary phase. Several types of HILIC columns are available, such as amino, diol-bonded, amide-bonded, or cyanopropyl columns as well as zwitterionic columns, but the most common used stationary phases are bare silica columns, especially when MS coupling is applied. The

retention can be influenced by the pH value of the mobile phase, therefore additives (usually formic acid, ammonium acetate, or ammonium formate) are added to the mobile phase, which improves the ionization of analytes in case of mass spectrometric detection. The retention (and ionization) of acidic analytes increases with increasing pH of the mobile phase and decreases for basic analytes [74, 77].

Examples for HILIC applications in the lipidomic practice are given in these papers [48, 58], where the lipid class separation according to the polar head groups was achieved, such as PE, LPE, PC, lysophosphatidylcholine (LPC), or SM. Other examples are shown in the published papers [78, 79], where HILIC mode is used for the identification and subsequent quantitation of glycosphingolipids.

Other chromatographic modes useful in lipidomic analysis

The following less common separation modes can provide a tailored selectivity for particular types of lipid isomerism: normal-phase chromatography (NP-HPLC), non-aqueous reversed phase chromatography (NARP), silver-ion chromatography (Ag-HPLC), chiral chromatography, or two-dimensional liquid chromatography (2D-LC).

NP-HPLC employs polar stationary phases (e.g., silicagel, aminopropyl, and cyanopropyl columns) and less polar mobile phases (heptan, hexan, or chloroform). The retention of analytes increases with their increasing polarity and decreases with increasing concentration of polar solution in the mobile phase [74]. NP-HPLC can be used for the separation of non-polar lipid classes, such as CE, cholesterol, TG, or DG [80]. To separate polar lipid classes, it is usually used a combination of polar stationary phase with the mixture of two solutions with different polarity (e.g., chloroform with acetonitrile and methanol) as the mobile phase.

NARP chromatography is a variation of RP-HPLC useful for the separation of very hydrophobic analytes, which are too strongly retained with conventional RP. In NARP mode, no water is used in the mobile phase, but usually mixtures of two solvents, one with higher polarity (acetonitrile or methanol) and one with lower polarity (chloroform, tetrahydrofuran, methylene chloride, or hexane/isopropanol). The retention decreases with decreasing ECN [71, 72, 74, 81, 82].

The principle of Ag-HPLC is based on the formation of weak silver ion complexes with double bonds (DB). The retention is influenced not only by the number of double bonds (higher number of DB = higher stability of silver ion complexes), but also by the distance of DB in the molecular structure, or by DB geometry, whereby *cis-* isomers form more stable silver ion complexes than *trans*-isomers. There are 3 approaches to enable silver ion complexation: adsorption of Ag-ions on the stationary phase, Ag-ions bonded via ionic bonds in the stationary phase, or addition of Ag-ions to the mobile phase, but this approach is not compatible with mass spectrometry detection due to ion suppression effects and the contamination of ion source by inorganic salts. Two types of mobile phases are frequently used, the first is based on chlorinated solvents with low concentration of polar modifiers (acetonitrile or methanol), and the second one is hexane-based mobile phase containing low concentration of acetonitrile as a polar modifier [77]. Silver-ion chromatography has been used mainly for the determination of TG and fatty acids [72, 83, 84].

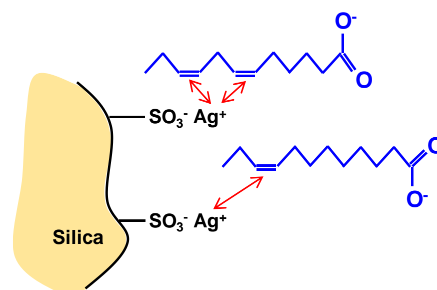


Figure 7 Principle of silver-ion chromatography.

Chiral chromatography is used for the separation of enantiomers (optical isomers). Their separation can be achieved by using chiral ligands bonded on a stationary phase based on cellulose or cyclodextrine), where analytes form reversible diastereomeric complexes with the chiral selector (one enantiomer has a higher affinity to the stationary phase than the other one), illustrated in **Figure 8.** Another possibility for the chiral separation is the addition of an enantiomeric pure chiral agent to the mobile phase, which forms diastereomeric complexes with the analyte. Chiral chromatography is very useful for the separation of TG enantiomers [73], for example in human plasma samples.

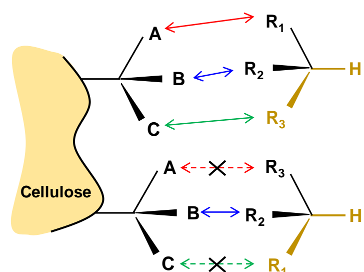


Figure 8 Principle of chiral chromatography

2D-LC uses a combination of two types of complementary chromatographic modes, e.g., HILIC mode for the lipid class separation, and RP mode for the lipid species separation either offline or online coupled. This approach was applied to the identification of 143 lipid species in human plasma and porcine brain [85].

1.2.3.2.2 Supercritical fluid chromatography

SFC is a separation technique, which is instrumentally similar to HPLC (**Figure 9**) [86] and combines the advantages of well-known chromatographic techniques: liquid chromatography (LC) and gas chromatography (GC). For SFC analysis, dedicated stationary phases can be used, but in general all stationary phases used for HPLC (polar and non-polar) can be applied for SFC as well. As the mobile phase, SFC uses a dense compressed gas, which is called supercritical fluid. The supercritical fluid region is reached once the temperature and pressure are above the critical temperature (T_c) and critical pressure (P_c) (**Figure 10**). Carbon dioxide (CO_2) is the most frequently used supercritical fluid due to low critical parameters (31.3°C and 73.7 bar) and safety reasons. Furthermore, $scCO_2$ is usually the first choice due to the low viscosity, the low surface tension, quite low price, the availability at high purity, non-toxicity, and non-flammability. $ScCO_2$ is of non-polar nature, therefore organic modifiers (methanol, ethanol, or isopropanol) can be added to the mobile phase to enhance the solvent strength and applicability range. Thanks to the low viscosity and high diffusion, it is possible to operate at high flow rates without high pressures, leading to high speed of analysis without the loss of separation efficiency. The addition of organic modifiers to CO_2 shifts the critical parameters, which may lead to the subcritical conditions instead of supercritical ones. In order to pressurize the system, a back-pressure regulator (BPR) after the column compartment is used in SFC. For connection of SFC to mass spectrometry, make-up solvents (usually methanol with additives) are added to the mobile phase to improve the continuous flow stability (no precipitation of analytes due to depressurized CO_2) and ionization efficiency [77].

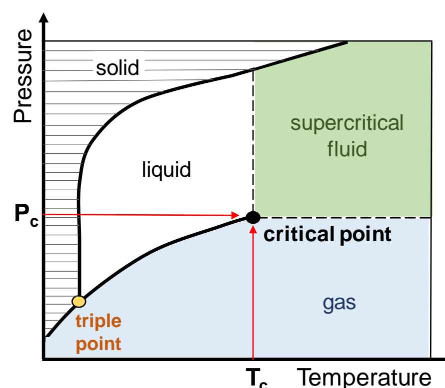


Figure 9 Phase diagram of carbon dioxide.

SFC has been a long time overshadowed by other separation methods, especially by HPLC. Recently, it was shown [58, 87] that SFC is a powerful tool in the lipidomic analysis, giving comparable results as established HPLC methods.

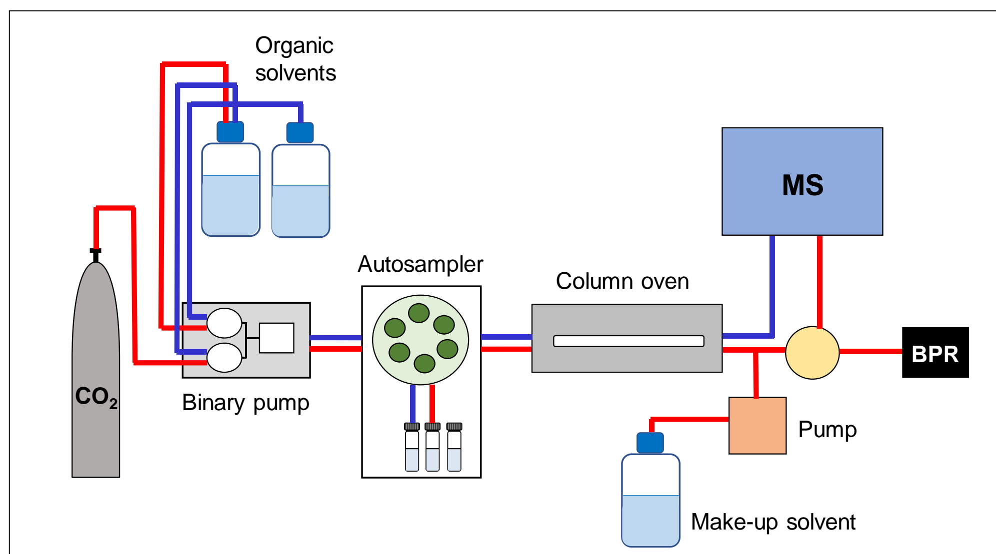


Figure 10 Scheme of HPLC (blue line) and SFC (red line) instrumentation.

1.2.3.3 Mass spectrometry

Chromatography coupled to mass spectrometry is the most popular approach for the lipidomic analysis. In mass spectrometry, neutral molecules are converted into ions (ionization), then separated according to *m/z* (mass-to-charge ratio) using mass analyzers based on different working principles, and measured their intensities using detectors [88].

MS measurement strategies usually depend on the abundance of lipid species in biological samples. Oxylipins or other low abundant lipid mediators are exclusively analyzed using targeted approaches, like selection reaction monitoring (SRM) [14, 16]. On the other hand, high abundant lipid species, such as glycerolipids or glycerophospholipids are usually analyzed using untargeted approaches, like full MS1 or MS/MS approaches [89].

The most universal and common ionization technique for MS coupled with chromatography is electrospray ionization (ESI), belonging to the soft ionization techniques, because the analyte degradation is less likely. Ions in ESI are transferred from the solution into the gaseous phase by applying electrical potential. A mobile phase and dissolved analytes in the mobile phase are brought to the ion source using metal capillary, to which a voltage (1 – 5kV) is applied. Charged droplets are generated at the exit of electrospray tip with the help of nebulizing gas (nitrogen). Solvents are evaporated using higher temperature and stream of drying nitrogen gas and then ions are ejected from the highly charged droplets followed by the acceleration into the mass analyzer [88]. ESI produces usually protonated (positive ion mode; $[M+H]^+$) or deprotonated (negative ion mode; $[M-H]^-$) ions or molecular adducts, such as $[M+Na]^+$, $[M+K]^+$, $[M+NH_4]^+$, or $[M+HCOO]^-$ and $[M+CH_3COO]^-$ in case of negative ion

mode. The observed adducts are mainly influenced by the analyte nature and the used additives in the mobile phase (type and concentration) [90]. Other ionization techniques are the atmospheric pressure chemical ionization (APCI) and the atmospheric pressure photoionization (APPI), especially suited for less polar mobile phases and analytes [88].

The best choice for accurate and highly sensitive quantitation applying MS/MS measurements (SRM scans, product ion scans, precursor ion scans, or neutral loss scans) are triple quadrupole (QqQ) instruments. Quadrupole one (Q1) and quadrupole three (Q3) operate independently and quadrupole two (Q2) works as a collision cell (with collision gas inside, usually argon or nitrogen), where the fragmentation of passed ions from Q1 occurs. In some hybrid variation of triple quadrupoles, Q3 can work as linear ion trap as well, which is called QTRAP [88]. To get higher mass accuracy of analytes, high resolution mass analyzers, such as quadrupole - time of flight (Q-TOF) analyzer or analyzers equipped by Fourier transformation (FT), such as Orbitrap or ion cyclotron resonance (ICR) are commonly used in the lipidomic research [88, 61, 66-69]. At recent time, the application of ion-mobility separation for the analysis of lipids gained popularity. Ion-mobility separation brings another dimension, where analytes are separated in a gas phase on the basis of different velocities in the electric field. The shape, the size, and the charge of analytes influences the collisional cross-section (CCS), which is characteristic for each molecule [91, 92]. The lipidome characterization can be also performed using desorption ionization techniques especially matrix-assisted laser desorption/ionization (MALDI). This approach is typically used for the analysis of lipids in tissue samples, allowing the determination of specific lipid distributions in a particular tissue area. However, quantitation with this approach is difficult due to extensive matrix effects and lower signal stability [93-96].

1.2.4 Quantitative lipidomics

There are different strategies of the lipidomic quantitation including the determination of relative and absolute concentrations of lipid species. Relative concentrations express the pattern changes of lipids in the lipidome (ratio between lipid species). Absolute concentrations express the total amount of lipids in a lipidome, for what using of IS is essential [97] and should be reported using molar concentrations. The type and number of IS are closely related to the selected approach for the lipidomic quantitation. An advantage of lipid class separation is the coelution of internal standard (IS) with endogenous analytes within the same lipid class, which guarantees the same matrix effect of IS and analytes allowing the accurate quantitation. A

limitation of this approach is that only the sum of number of carbons (CN) and double bonds (DB) of lipids can be determined without any detailed structural information of the fatty acyl chains, what is the advantage of lipid species separation. In case of lipid species separation, the lipid structure is typically characterized in more details than for lipid class separation, but the IS have to be carefully selected, because of different matrix effects and ion suppression within separation run. Shotgun MS approach is similar to the lipid class separation with regard to the quantitation, as the IS and the analytes of the lipid class coelute within characteristic scan events. For accurate and sensitive quantitation, it is important to optimize the used lipid concentration, solvents, and additives of the infusate for improved ionization efficiency and consider that the lipid structure as well as isobaric and isotopic overlaps may influences the lipid response (the same as for the lipid class separation approach) [7, 59].

1.2.4.1 Internal standards

The use of appropriate IS is a prerequisite for any MS-based quantitation including the lipidomic analysis. The mixture of IS has to be added to the real samples before extraction and their concentrations should be close to physiological values. This way, it is guaranteed that the analytes and IS are influenced in the same way during the sample preparation and analysis with regard to recovery rate, matrix effect, or ion suppression. The structural similarity, the exogenous nature, the fragmentation behavior, the concentration, and the analytical response should be considered for the selection of IS. The best approach would be to use stable isotopically labeled analogues of each lipid species with known concentration of IS. However, a limited range of isotopically labeled IS is available on the market, and costs for the synthesis of hundreds of isotopic labeled analogues of natural occurring lipids in biological samples are not be affordable. Therefore, it is recommended to use as a minimum requirement one IS per class for the quantitative lipidomic analysis using lipid class separation approach. However, it is very useful to use more IS per class, as the quantitation error can be determined by mutual quantitation of the IS with known concentrations. For the lipid species separation, it is recommended to use much more lipid IS to cover the whole chromatographic run. It is recommended to report, which IS was used for the quantitation of specific lipid species. In lipidomic analysis, exogenous IS typically have shorter, longer fatty acyl chains, odd number of carbons, or isotopically labeled lipid species (e.g., ^{13}C, D) [8, 97].

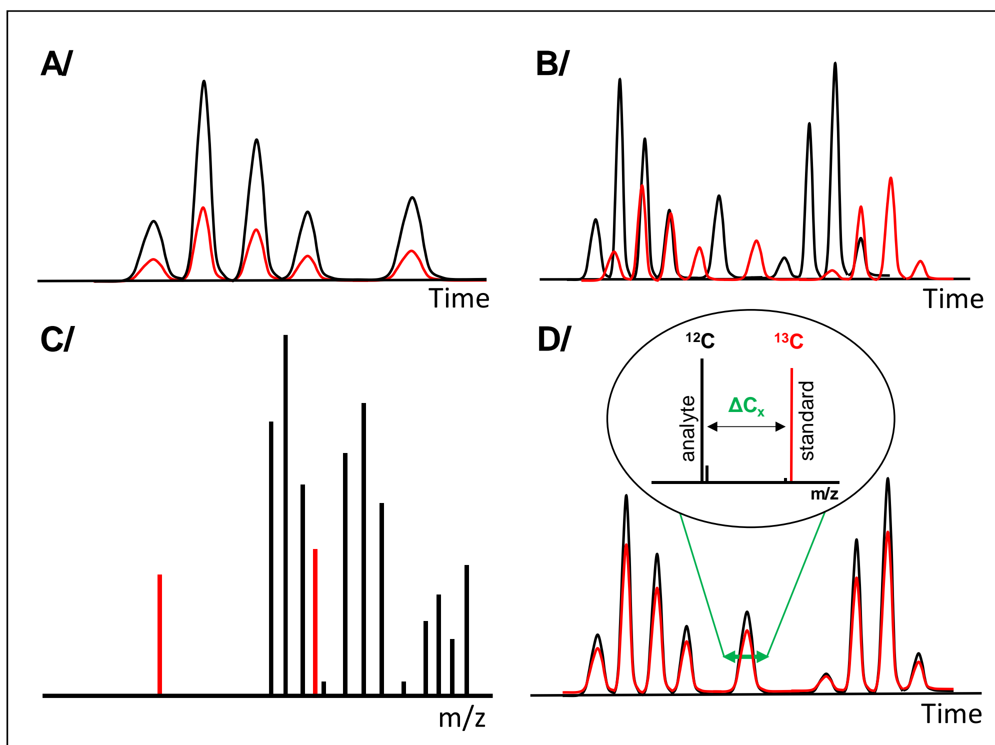


Figure 11 The quantitation approaches. **A/** lipid class separation with at least 1 IS per lipid class; **B/** lipid species separation using more ISs to cover the separation run; **C/** shotgun MS using 2 IS for lipid class (characteristic scan event); **D/** the best quantitation approach using ^{13}C isotopically labeled lipid species as internal standards. Red line – internal standards, black line – endogenous lipids.

1.2.4.2 Quality control measurements and validation

Special attention should be paid to monitor and confirm precision, accuracy, and quality of the quantitative lipidomic method to ensure high data quality. A simple way to verify good instrumental performance is the regular measurements of QC samples [98]. 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. QC samples can be divided into two groups. The first group is called as batch quality control (BQC), important for monitoring equal quality of individual batches (and potentially compensate the variations between individual batches), extremely momentous, when extractions are performed during several days. BQC samples should always be prepared from the same sample source within a study but extracted at different times (depends on the size of batches). Individual batches should provide reproducible results with minimum errors. The second group is called total QC (TQC), which is important to monitor the performance of analytical platforms over time. This type of QC can be prepared for instance by mixing aliquots of BQC samples, then aliquots of this pooled sample are prepared and adequately stored. In this way, it is guaranteed

that always the same TQC sample is measured within the study. It is common at MS-based methods to lose sensitivity during measurements of hundreds of samples with complex matrix due to the gradual contamination of mass spectrometer. TQC samples can help to evaluate the extent of the signal drop and based on that interventions are performed, e.g., cleaning of the ion source [8].

Blank samples (without matrices, such as plasma or serum) should be prepared, using the same materials and solvents as for the study samples. Blank samples allow the evaluation of the presence of impurities or interferences with the same *m/z* as the target analytes, so they should be regularly measured within the sequence together with QC samples. Other options of quality control monitoring are to measure calibration curves and dilution series during the sequence. The calibration curve provides information about a signal saturation, which is connected to the accurate quantitation, because each analyzed lipid species should provide a linear response. Dilution series (for example dilution of QC sample) can prevent a misidentification of lipid species due to the fact that all lipids should decrease their response with higher dilution [8].

The NIST SRM 1950 plasma (lithium heparin plasma) is a standard reference material, which was prepared in 2007 from 100 healthy female and male volunteers (1:1) representing the ethnic distribution in the United States [26, 99, 100]. This reference material was analyzed by 31 laboratories (using different approaches and instruments) from different countries and afterwards consensus values for determined lipids (molar concentrations of lipids) were reported [26]. The analysis of NIST plasma can be useful to obtain valuable information about the variability across the analytical approaches (within one and more laboratories), the instrumental performance, the quantitation bias, the data processing software, or to discover problematic lipid species providing inconsistent quantitation data. Therefore, it should be regularly measured together with QC and blank samples within the study. With the lipid concentrations obtained for the NIST plasma, the lipid concentrations of the real samples can be standardized and normalized in order to get more consistent data within the lipidomics community, important for the future integration of lipidomics in clinical chemistry laboratories with reference ranges of concentrations[8].

Another point to get reliable results and to avoid errors as much as possible is the randomization of samples [8]. Samples should be randomized for sample preparation, measurements, and for data processing as well.

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) [101] or the European Medical Agency (EMA) [102]. These guidelines were not developed for the lipidomic analysis, but initially for pharmaceutical applications. However, most of the criteria and strategies are applicable for the validation of lipidomic methods as well. The main criteria of mentioned guidelines are calibration curve, limit of quantitation (LOQ) or lower limit of quantitation (LLOQ), carry-over effect, recovery rate, matrix effect, precision and accuracy (inter- and intra-day), stability, repeatability, selectivity, dilution integrity, and additional criteria, such as process efficiency and extraction yield. The whole validation process has to be performed using internal standards and the real biological matrices (usually a pooled sample; only parameters, such as matrix effect and selectivity has to be determined using the individual biological samples in line with guidelines).

1.2.4.3 Data processing and statistical evaluation

Another key factor of lipidomic analysis is the data processing. The applied data processing method, software, and used parameters should be accurately described in published papers for readers to allow the future repeatability. Data processing obtained from MS-based methods strongly depends on used methods and instrumentations (targeted versus untargeted approaches, low resolution versus high resolution mass analyzers, etc.). Moreover, individual instrument vendors have different raw file formats and their own data processing tools to get ion mass-signal response (areas or intensities). For the accurate quantitation, the isotopic correction should be performed, especially for lipid class separation methods (e.g., HILIC-UHPLC/MS) and direct infusion/MS methods, where the intensity of lipids are may affected from isotopomers from lipid species with one double bond more. For lipid species separation approaches, especially in combination with ultra-high resolution mass spectrometry, no isotopic correction is needed due to complete MS resolution of all common isotopomers [7].

Lipidomic data may be filtered by setting certain criteria, e.g., the exclusion of lipid species for a RSD>20% of all measured QC samples within the study [8].

A statistical evaluation is important in lipidomic analysis as well, especially for bioanalytical research. The statistics helps to better understand and explore the behavior of lipids in biological systems (cell, organism, or human body). Quite common statistical plots used in lipidomic research are box plots, volcano plots, or correlation plots. For the visualization of differences between groups (in clinical research usually differences in lipidome between healthy humans and patient suffering from particular disease), the principal component analysis

(PCA) and the orthogonal partial least squares discriminant analysis (OPLS-DA) belong to the most popular ones. Other common plots that show regulations of lipids between two sample groups are S-plots and heatmaps [48-50]. There are many statistical tools either for free or paid on the market, which can be useful for better understanding and visualization of observed results.

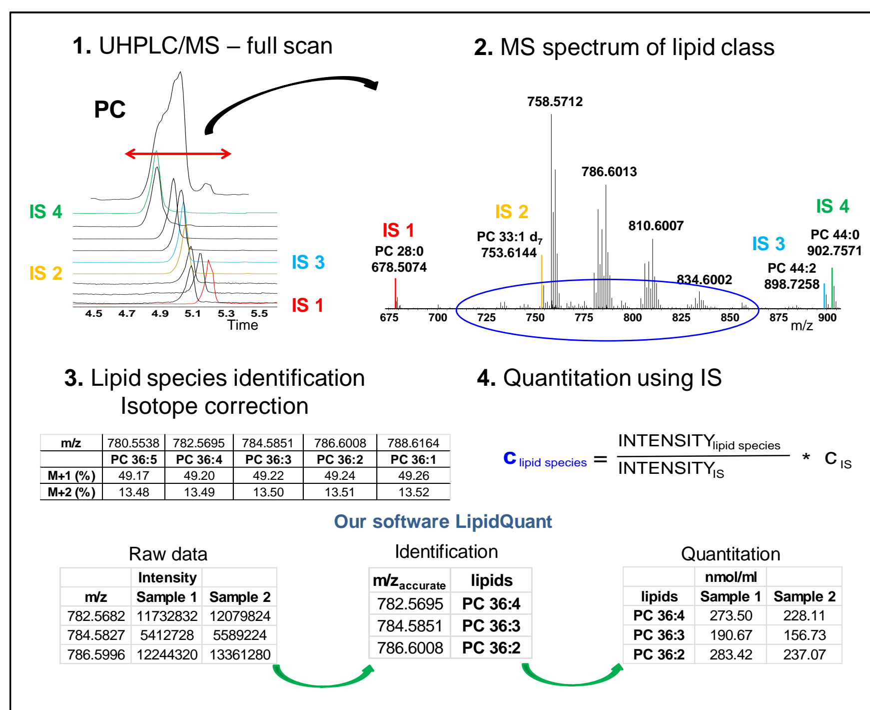


Figure 12 Principle of quantitation of HILIC-UHPLC/MS method in our laboratory.

1.2.5 Applications

Metabolomics (including lipidomics) aims the exploration of the metabolome (lipidome) and clarification of biological functions of metabolites produced by cells, tissues, or the whole organism. Changes in the genome, the transcriptome, and the proteome are finally reflected in the metabolome (the central dogma of molecular biology). Changes in the metabolome and the metabolomic activity can be caused by endogenous factors but also by exogenous factors, such as environmental factors, the internal microbiome, oxidative stress, pathogens, pollutants, inflammation, lifestyle, medicaments, drugs, food, etc., which defines our phenotype. Several diseases, such as diabetes, heart diseases, obesity, neurodegenerative diseases, and cancer, have a dominant metabolomic component (especially they have a close connection with lipids) and understanding of mechanism can significantly improve the

metabolomic disorders knowledge [76], therefore an attention is paid to metabolomic and lipidomic studies.

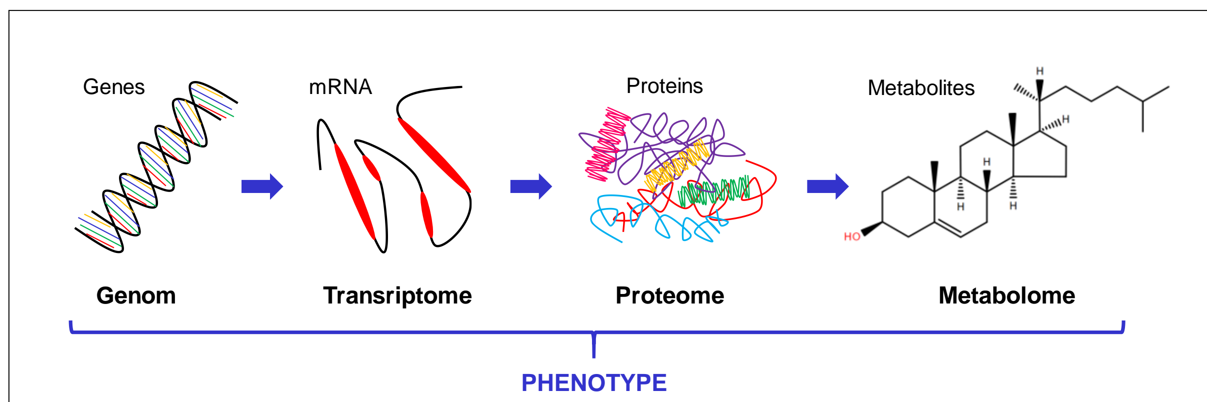


Figure 13 The central dogma of molecular biology.

2 Aims of the thesis

The present Ph.D. thesis is focused on quantitative approaches of lipidomic analysis using chromatography coupled to mass spectrometry. Lipid species separation (RP-UHPLC/MS) and lipid class separation achieved using HILIC-UHPLC/MS and UHPSFC/MS bring the own requirements for lipid quantitation shown in this work. MS methods with prior chromatography were optimized, validated, and applied to the measurements of the set of biological samples. The goal of this work is to present possibilities of high-throughput analysis of lipids, such as oxylipins, glycerolipids (MG, DG, and TG), CE, Cer, SM, PC, and LPC in biological samples and applications of lipidomics to the clinical studies with recommendations of quality control measurements. There is also shown the analysis of GSL in biological samples using HILIC-UHPLC/MS, but with my partial contribution.

A. Overview of mass spectrometric approaches for lipidomic quantitation

- Described in Section 3.1 and appendix I.
- 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

- Described in Section 3.2 and appendix II.
- 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

- Described in Section 3.3 and appendix III., IV., V., and VI.
- 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)

- Described in Section 3.4 and appendix VII. and VIII.
- Determination of GSL using HILIC-UHPLC/MS and application to measurements of the biological samples

3 Results and discussion

Published papers and manuscripts representing the core of this Ph.D. thesis are put into the context in this section. Full versions of all manuscripts are attached in the appendix section of this work.

3.1 Overview of mass spectrometric approaches for lipidomic quantitation

Lipidomics is involved in clinical studies, such as cancer research (oncolipidomics) to find regulated lipids useful for future diagnosis purposes with the intention to develop robust and high-throughput methodologies for the quantitation of lipids in biological samples, such as body fluids, tissues, and cell lines. Quantitative lipidomics approaches are based on MS analysis. The main three methodologies either coupled to chromatography, direct infusion (shotgun MS), or desorption ionization and mass spectrometry imaging (MSI) are described with quantitation approaches, principles, data processing requirements, the emphasis of the quality control measurements, and the method validation significant for the reliable quantitation. The lipid classification and nomenclature important for the harmonization of lipid species information obtained from MS-data is discussed with example of the shortland notation. Furthermore, the description of the most common biological materials is presented together with the possibilities of the sample preparation protocols and other spectroscopic methods applicable in lipidomic analysis.

A special attention was payed to the critical 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. Similar regulation trends of lipids were observed, what is a positive start for possible future screening of multiple cancers by lipidomic analysis. However, some discrepancies were discovered, which could be explained by inconsistencies in quantitation approaches, underestimation of the method validation, QC monitoring, improper selection of IS, and mixed lipid annotation styles. These facts prove an exigency of lipid analysis harmonization with requirements for lipidomic analysis.

Reference

Appendix **I.**

Wolrab D., Jirásko R., **Chocholoušková M.**, Peterka O., Holčapek M. Oncolipidomics: Mass spectrometric quantitation of lipids in cancer research. *TrAC* **120** (2019) 115480. (Q1/D1; IF=9.801)

3.2 Lipid species separation of oxylipins using reversed-phase ultrahigh performance liquid chromatography: optimization, validation, and application to human plasma samples

Polyunsaturated fatty acids (PUFA) generate (either by enzymatic or non-enzymatic pathways) hundreds of bioactive lipid species called oxylipins. Their structures are very similar, which makes their analysis quite challenging together with their low concentrations in biological samples, for example in human plasma or serum. Lipid species separation using reversed-phase liquid chromatography coupled to triple quadrupole (QqQ) mass spectrometry using SRM scans is the most convenient way to comprehensively and quantitatively analyze oxylipin isomers.

The separation of oxylipins was performed on C18 column (150 x 2.1 mm, 1.7 µm) with 12 min run time. Oxylipins were measured in the negative-ion mode and parameters of SRM method, such as collision energy, precursor ions, and main product ions, were optimized using 63 oxylipins standards to obtain the highest sensitivity. Due to the low abundance of oxylipins in human plasma samples, a solid phase extraction was used for the sample preparation. The developed method was validated for parameters, such as calibration curve, carry-over, precision, accuracy, recovery rate, matrix effect to evaluate reliability of method and afterwards applied for a small pilot study of 40 female human plasma samples of breast cancer patients (20) and healthy controls (20).

Analyzed oxylipins in samples were quantified using 14 deuterated IS of oxylipins added before the extraction with reported information, which IS was used for the quantitation of individual determined oxylipins. We were able to quantify 21 oxylipin species, and 36 oxylipin species were detected in total. Our determined concentrations of healthy volunteers were in agreement with literature values.

Lipidomic data were statistical evaluated using *p*-values, *T*-values, variable importance in projection (VIP), unsupervised PCA, supervised OPLS-DA, S-plot, and box plots. 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.

Reference

Appendix **II.**

Chocholoušková M., Jirásko R., Vrána D., Gatěk J., Melichar B., Holčapek M. Reversed phase UHPLC/ESI-MS determination of oxylipins in human plasma: a case study of female breast cancer. *Anal. Bioanal. Chem.* **411** (2019) 1239-1251. (Q1; IF=3.637)

3.3 Lipid class separation approaches (HILIC-UHPLC/MS and UHPSFC/MS): optimization, validation, and application to set of samples of biological samples

Another quantitative approach in the lipidomic analysis is the lipid class separation achieved by HILIC-UHPLC/MS or UHPSFC/MS methods. First, efforts were put into the optimization of sample preparation and the full validation important for the accurate quantitation. These methods were used for analyses of lipids, such as glycerolipids (MG, DG, and TG), CE, Cer, SM, LPC, and PC, which are more abundant in biological samples than oxylipins, therefore liquid-liquid extraction was sufficient for the sample preparation. Six extraction protocols were compared, and extraction additives and dissolution solvents for extract were optimized. Modified Folch approach (double extraction) was evaluated as the best extraction procedure with regard to recovery rate, matrix effect, and precision with adding of ammonium carbonate as an additive, and mixture of chloroform/methanol (1:1) as the dissolution solvent. Afterwards, both methods were validated for these parameters: calibration curve, lower limit of quantitation (LLOQ), selectivity, carry-over, dilution integrity, extraction recovery, process efficiency, matrix effect, extraction yield, and especially for accuracy and precision in order to compare an applicability of UHPSFC/MS to well-established HILIC-UHPLC/MS technique for the study of biological samples. The validation of both methods was performed using the same sample set of 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, which brings advantages of separation of non-polar and polar lipid classes (unlike to HILIC-UHPLC/MS), shorter separation run, and slightly high robustness. The application of IS (deuterated and exogenous) for individual lipid classes is a key element for reliable lipidomic quantitation. Their selection was performed according to the selectivity and the calculation of quantitation error. The validated methods were applied to the NIST Standard Reference Material 1950 human plasma to compare determined lipid concentrations obtained from both methods to literature values (Appendix **III.**).

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. In total, 171 lipid species and 122 lipid species were quantified using UHPSFC/MS and HILIC-UHPLC/MS, respectively and 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. The special emphasis is given to the comparison of HILIC-UHPLC/MS and UHPSFC/MS based on the determination of lipid concentrations, where some differences were found, which can be compensated by the normalization using the NIST Standard Reference Material 1950 human plasma. Based on this study, recommendations for the quality of measurements including the randomization, the monitoring of the instrument performance, the procedure for the sample preparation using appropriate QC, and using more than one IS per lipid class are discussed (Appendix **IV.**).

Another example of the application of these methods (together with MALDI) is the lipid characterization of exosomes isolated from human plasma of 12 healthy volunteers. The lipidomic composition and lipid concentrations of exosomes were compared on the basis of used methods and with plasma needed for their isolation to highlight differences. 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 (Appendix **V.**).

The special importance is given to the investigation of robustness of developed methods, therefore HILIC-UHPLC and UHPSFC were connected to two different QTOF mass spectrometers from the same vendor. The comparison of 4 platforms were based on

measurements of lipid concentrations in the same samples of the set of healthy volunteers and kidney cancer patients, where the final observations are discussed, and the same final outputs of differentiation of cancer and control groups are observed. The same extracts, the same chromatography parameters, the same sequences, and the same data processing were used in all cases to keep the identical conditions. This experiment showed that even measurements of the same extracts in the same laboratory by identical chromatography methods, but differing only mass spectrometers, can produce some differences in lipid concentrations. However, these concentration differences can be normalized using the NIST Standard Reference Material 1950 human plasma to significantly reduce variations among data sets, what is an important step towards the determination of true lipid concentrations independently on used quantitative approach and used instruments (Appendix **VI.**).

References

Appendix **III.**

Wolrab D., **Chocholoušková M.**, Jirásko R., Peterka O., Holčapek M. Validation of lipidomic analysis of human plasma and serum by supercritical fluid chromatography-mass spectrometry and hydrophilic interaction liquid chromatography-mass spectrometry. *Anal. Bioanal. Chem.* **412** (2020) 2375-2388. (Q1; IF=3.637)

Appendix **IV.**

Wolrab D., **Chocholoušková M.**, Jirásko R., Peterka O., Mužáková V., Študentová H., Melichar B., Holčapek M. Determination of one-year stability of lipid plasma profile and comparison of blood collection tubes using UHPSFC/MS and HILIC-UHPLC/MS. *Anal. Chim. Acta*, revision submitted. (Q1; IF=5.977)

Appendix **V.**

Peterka O., Jirásko R., **Chocholoušková M.**, Kuchař L., Wolrab D., Hájek R., Vrána D., Strouhal O., Melichar B., Holčapek M. Lipidomic characterization of exosomes isolated from human plasma using various mass spectrometric techniques. *BBA- Mol. Cell Biol. L.* **1865** (2020) 158634. (Q1; IF=4.519)

Appendix **VI.**

Chocholoušková M., Wolrab D., Jirásko R., Študentová H., Melichar B., Holčapek M. Intra-laboratory cross-comparison of four lipidomic quantitation platforms using hydrophilic interaction liquid chromatography vs. supercritical fluid chromatography coupled to two quadrupole - time-of-flight mass spectrometers. *Talanta*, final editing before submission. (Q1; IF=5.339)

3.4 Lipid class separation (HILIC-UHPLC/MS) of glycosphingolipids in biological samples (partial contribution)

GSL are a heterogeneous class of lipids with the connection to several diseases including cancer. Their determination in biological samples is 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. The suitability of several C18 based SPE columns were compared. 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 (Appendix **VII.**). The subsequent work is in progress with the full validation and application to bigger set of clinical samples.

Body fluids are not only biological samples suitable for lipidomic analysis. The lipid composition can be also determined in other biological samples, such as hippocampus of mice to investigate forebrain synaptic junctions. Gangliosides are subclass of GSL measured using HILIC-UHPLC/MS as well, which was my contribution to the submitted paper. The analysis of gangliosides was one of the multi-omics strategies to determine the general molecular composition of forebrain synaptic junctions (Appendix **VIII.**).

References

Appendix **VII.**

Hořejší K., Jirásko R., **Chocholoušková M.**, Kahoun D., Holčapek M. Structural characterization of glycosphingolipids in human plasma using hydrophilic interaction

chromatography – electrospray ionization mass spectrometry. *Anal. Chim, Acta*, submitted. (Q1; IF=5.977)

Appendix **VIII.**

Borgmeyer M., Coman C., Has C., Schott H.-F., Li T., Westhoff P., Cheung Y.F.H., Hoffman N., Yuanxiang P., Behnisch T., Gomes G.M., Dumenieu M., Schweizer M., **Chocholoušková M.**, Holčapek M., Mikhaylova M., Kreutz M.R., Ahrends R. Multiomics characterization of synaptic junctions and lipid signaling in enriched environment. *Nat. Commun.*, submitted. (Q1/D1; IF=12.121)

4 Conclusions

This Ph.D. thesis focused on a lipidomic analysis using MS coupled to chromatography and represents examples of 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. SRM method was developed for 63 oxylipins, validated, and applied to the measurements of the small study of clinical samples of healthy volunteers and breast cancer patients to find potential for diagnosis purposes. The quantitation was performed using 14 deuterated IS of oxylipins, which covered the chromatographic run. IS were added before SPE sample preparation needed for the enrichment of oxylipins in human plasma.

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, which are higher abundant in comparison to oxylipins, therefore optimized liquid-liquid extraction protocol was used without any enrichment steps (modified Folch procedure). 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 with the possibility of data normalization in case of lipid concentration changes. For quantitation purposes, we used at least two IS (deuterated and exogenous) per lipid class added before the extraction.

HILIC-UHPLC/MS method is also useful for the determination of 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. Another example was the application of HILIC-UHPLC/MS method for the quantitation of gangliosides in biological samples, which was used for the multi-omics characterization the general molecular composition of forebrain synaptic junctions.

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Scientific contribution

Published papers in impact journals related to Ph.D. thesis

1. Wolrab D., Jirásko R., **Chocholoušková M.**, Peterka O., Holčapek M. Oncolipidomics: Mass Spectrometric Quantitation of Lipids in Cancer Research. *TrAC* **120** (2019) 115480. (Q1/D1; IF=9.801)
2. **Chocholoušková M.**, Jirásko R., Vrána D., Gatěk J., Melichar B., Holčapek M. Reversed-phase UHPLC/ESI-MS determination of oxylipins in human plasma: case study of female breast cancer. *Anal. Bioanal. Chem.* **411** (2019) 1239-1251. (Q1; IF=3.637)
3. Wolrab D., **Chocholoušková M.**, Jirásko R., Peterka O., Holčapek M. Validation of lipidomic analysis of human plasma and serum by supercritical fluid chromatography-mass spectrometry and hydrophilic interaction liquid chromatography-mass spectrometry. *Anal. Bioanal. Chem.* **412** (2020) 2375-2388. (Q1; IF=3.637)
4. Peterka O., Jirásko R., **Chocholoušková M.**, Kuchař L., Wolrab D., Hájek R., Vrána D., Strouhal O., Melichar B., Holčapek M. Lipidomic characterization of exosomes isolated from human plasma using various mass spectrometric techniques. *BBA- Mol. Cell Biol L.* **1865** (2020) 158634. (Q1; IF=4.519)

Submitted papers in impact journals related to Ph.D. thesis

1. Wolrab D., **Chocholoušková M.**, Jirásko R., Peterka O., Mužáková V., Študentová H., Melichar B., Holčapek M. Determination of one year stability of lipid plasma profile and comparison of blood collection tubes using UHPSFC/MS and HILIC-UHPLC/MS. *Anal. Chim. Acta*, revision submitted. (Q1; IF=5.977)
2. **Chocholoušková M.**, Wolrab D., Jirásko R., Študentová H., Melichar B., Holčapek M. Intra-laboratory cross-comparison of four lipidomic quantitation platforms using hydrophilic interaction liquid chromatography vs. supercritical fluid chromatography coupled to two quadrupole - time-of-flight mass spectrometers. *Talanta*, final editing before submission. (Q1; IF=5.339)
3. Hořejší K., Jirásko R., **Chocholoušková M.**, Kahoun D., Holčapek M. Structural characterization of glycosphingolipids in human plasma using hydrophilic interaction

chromatography – electrospray ionization mass spectrometry. *Anal. Chim. Acta*, submitted. (Q1; IF=5.977)

4. Borgmeyer M., Coman C., Has C., Schott H.-F., Li T., Westhoff P., Cheung Y.F.H., Hoffman N., Yuanxiang P., Behnisch T., Gomes G.M., Dumenieu M., Schweizer M., **Chocholoušková M.**, Holčapek M., Mikhaylova M., Kreutz M.R., Ahrends R. Multiomics characterization of synaptic junctions and lipid signaling in enriched environment. *Nat. Commun.*, submitted. (Q1/D1; IF=12.121)

Paper in impact journal not related to Ph.D. thesis

1. **Chocholoušková M.**, Komendová M., Urban J. Retention of small molecules on polymethacrylate monolithic capillary columns. *J. Chromatogr. A* **1488** (2017) 85-92. (Q2; IF=4.049)

Posters – international conferences

1. Peterka O., Jirásko R., **Chocholoušková M.**, Wolrab D., Kuchař L., Holčapek M. Lipidomic analysis and comparison of human body fluids and exosomes by various MS techniques. **HPLC 2019 Milano**, Italy, 16. – 20.6. 2019.
2. **Chocholoušková M.**, Wolrab D., Jirásko R., Vrána D., Melichar B., Holčapek M. HILIC-UHPLC/MS for quantitative lipidomics: An optimized and validated method for the analysis of body fluids. **HPLC 2019 Milano**, Italy, 16. – 20.6. 2019.
3. **Chocholoušková M.**, Wolrab D., Holčapek M. Analysis of lipids using hydrophilic interaction chromatography coupled to mass spectrometry: optimization of sample preparation and validation. **Mass Spectrometry Forum 2019**, Vienna, Austria, 19.2. – 20.2. 2019.
4. **Chocholoušková M.**, Jirásko R. Holčapek M. Determination of oxylipins in clinical samples using UHPLC/MS. **Lipidomics Forum 2018,** Dortmund, Germany, 11. – 13.11.2018.
5. **Chocholoušková M.**, Jirásko R., Holčapek M. UHPLC/MS determination of oxylipins in clinical samples. **ASMS 2018,** San Diego, USA, 3. – 7.6.2018.
6. **Chocholoušková M.**, Cífková E., Holčapek M. Analysis of Eicosanoids in biological samples using the coupling of separation techniques and mass spectrometry. **HPLC 2017,** Prague, Czech Republic, 18. – 22.6.2017.

Posters – national conferences

1. **Chocholoušková M.**, Wolrab D., Jirásko R., Vrána D., Melichar B., Holčapek M. Quantitation of lipids using HILIC-UHPLC/MS: An optimized and validated method for the analysis of biological samples. **School of mass spectrometry 2019**, Špindlerův mlýn, Czech Republic, 8.9. – 13.9. 2019.
2. Peterka O., **Chocholoušková M.**, Jirásko R., Wolrab D., Hájek R., Kuchař L., Holčapek M. Lipidomic comparison of exosomes and human plasma by mass spectrometry. **School of mass spectrometry 2019**, Špindlerův mlýn, Czech Republic, 8.9. – 13.9. 2019.
3. **Chocholoušková M.**, Jirásko R., Holčapek M. Determination of oxylipins in human plasma samples by UHLPC/MS. **School of mass spectrometry 2018,** Špindlerův Mlýn, Czech Republic, 9. – 14.9.2018.

Lectures – international conferences (co-author)

1. Holčapek M., Wolrab D., Jirásko R., Cífková E., Peterka O., **Chocholoušková M.**, Brabcová I., Vrána D., Melichar B., Hrstka R. Lipid cancer biomarkers in early diagnosis: where we are now and what are future steps, **32nd International Symposium on Chromatography (ISC)**, Cannes, France, 23. - 27. 9. 2018.
2. Holčapek M., Wolrab D., Jirásko R., Cífková E., Peterka O., **Chocholoušková M.**, Brabcová I., Vrána D., Melichar B., Hrstka R. Lipidomic Analysis in Cancer Biomarker Research, Torun, Poland, 26. - 28. 10. 2018.
3. Wolrab D., **Chocholoušková M.**, Jirásko R., Peterka O., Holčapek M. UHPSFC/MS: A powerful Tool for the Lipid Analysis of Biological samples, **30th MassSpec Forum**, Vienna, Austria, 19 - 20. 2. 2019.
4. Holčapek M., Wolrab D., Jirásko R., **Chocholoušková M.**, Peterka O., Vrána D., Melichar B., Hrstka R. High-throughput Lipidomic Profiling of Human Blood: Potential for Early Screening of Multiple Cancer Types, **HPLC 2019**, Milano, Italy, 16. - 20. 6. 2019.
5. Wolrab D., **Chocholoušková M.**, Peterka O., Jirásko R., Hrstka R., Vrána D., Melichar B., Holčapek M. Quantitative Lipidomic Analysis of Biological Samples: Optimization and Application for the Analysis of Clinical Samples using UHPSFC/MS, **HPLC 2019**, Milano, Italy, 16. - 20. 6. 2019.

6. Holčapek M., Wolrab D., Jirásko R., **Chocholoušková M.**, Peterka O. High-throughput Lipidomic Quantitation of Biological Samples, **15th Annual Conference of the Metabolomics Society**, Hague, Netherlands, 23. – 27. 6. 2019.
7. Holčapek M., Wolrab D., Jirásko R., **Chocholoušková M.**, Peterka O. High-throughput Lipidomic Quantitation of Biological Samples, **25th International Symposium on Separation Sciences**, Lodz, Poland, 15. – 18. 9.2019.
8. Holčapek M., Wolrab D., Jirásko R., **Chocholoušková M.**, Peterka O. Applied Lipidomics: Quantitative Approaches and Cancer Applications, **Opening of the Center for Explorative Lipidomics**, Graz, Austria, 25. 9. 2019.
9. Holčapek M., Wolrab D., Jirásko R., **Chocholoušková M.**, Peterka O. Quality Assurance and Quality Control in the Lipidomic Quantitation Using Lipid Class Separation – Mass Spectrometry, **Lipidomics Forum 2019**, Borstel, Germany 10. - 12.11.2019.
10. Wolrab D., **Chocholoušková M.**, Holčapek M. UHPSFC/MS: A powerful Tool for the Analysis of Biological Samples, **2nd STARSS conference on Separation Science**, Hradec Králové, Czech Republic, 25. – 27.11.2018.

Lectures – national conferences (co-author)

1. Holčapek M., Wolrab D., Jirásko R., Cífková E., Peterka O., **Chocholoušková M.**, Brabcová I., Vrána D., Melichar B., Hrstka R., Lipidomická analýza hmotnostní spektrometrií ve screeningu nádorových onemocnění, **School of mass spectrometry 2018**, Špindlerův Mlýn, Czech Republic, 9. - 14. 9. 2018.
2. Holčapek M., Wolrab D., Jirásko R., **Chocholoušková M.**, Peterka O. Kvantitativní přístupy v lipidomice, **School of mass spectrometry 2019**, Špindlerův Mlýn, Czech Republic, 9. – 13. 9. 2019.
3. Holčapek M., Jirásko R., **Chocholoušková M.**, Nováková L. Vývoj LC-MS metod pro malé molekuly, **School of mass spectrometry 2019**, Špindlerův Mlýn, Czech Republic, 9. – 13. 9. 2019.

Research intership

1. **National University of Singapore**, Department of Biochemistry, Singapore Lipidomics Incubator (SLING), **Supervisor:** Dr Amaury C. Gassiot, 1.10. – 31.12. 2019.

Awards

1. **Best poster award: Chocholoušková M.**, Jirásko R., Holčapek M. Determination of oxylipins in human plasma samples by UHLPC/MS. **School of mass spectrometry 2018,** Špindlerův Mlýn, Czech Republic, 9. – 14.9.2018.
2. **Best poster award (co-author):** Peterka O., **Chocholoušková M.,** Jirásko R., Wolrab D., Hájek R., Kuchař L., Holčapek M. Lipidomic comparison of exosomes and human plasma by mass spektrometry. **School of mass spectrometry 2019**, Špindlerův mlýn, Czech Republic, 8.9. – 13.9. 2019.

Attended courses

1. **Metabolomic analysis,** lecturer: T. Hankemeier, HPLC 2017 Praha, 18. – 22.6.2017.
2. **Lipidomic analysis,** lecturer: G. Liebisch, M. Holčapek, HPLC 2017 Praha, 18. – 22.6.2017.
3. **Interpretation of EI spectra,** lecturer: M. Polášek, J. Cvačka, M. Holčapek, J. Čáslavský, School of mass spectrometry 2017, Luhačovice, Czech Republic, 10. – 11.9.2017.
4. **Troubleshooting in LC-MS,** lecturer: L. Nováková, School of mass spectrometry 2018 Špindlerův Mlýn, Czech Republic, 9.9.2018.
5. **Workflow in metabolomics,** lecturer: I. Kohler, School of mass spectrometry 2018 Špindlerův Mlýn,10.9.2018.
6. **Development of LC-MS methods for small molecules,** lecturer: M. Holčapek, L. Nováková, School of mass spectrometry 2019 Špindlerův Mlýn, 8.9.2019.
7. **Statistical data analysis,** lecturer: D. Friedecký, School of mass spectrometry 2019 Špindlerův Mlýn, 8.9.2019.
8. **Introduction of GLP regulations and bioanalytical method validation by LC-MS,** lecturer: P. Wang, HPLC 2019, Milano, Italy, 16.6.2019.

9. **Statistical analysis of chromatographic data: a practical guide,** lecturer: G. V. Truyols, HPLC 2019, Milano, Italy, 16.6.2019.
10. **Summer school LipoSysMed – Integration of large scale lipidomics data in systems medicine research,** Leipzig, Germany, 18.3. – 22.3. 2019.