**Palacký University Olomouc**

# Master’s thesis

**Olomouc 2015** **Markéta Machálková**

**Palacký University Olomouc**

**Faculty of Science**

**Department of Cell Biology and Genetics**

# LC-MS analysis of chosen phosphatidylcholines in human plasma

**Master's Thesis**

**Markéta Machálková**

Study programme: Biology
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**Advisor: Mgr. Ivo Vrobel**

I hereby declare that I wrote this thesis solely by myself and that all sources used in this thesis are cited and included in the references part.

In Olomouc …….

# Souhrn

Alzheimerova choroba je vážné neurodegenerativní onemocnění, které je charakterizováno ztrátami paměti a neschopností samostatně vykonávat běžné činnosti. Ačkoliv lék na tuto nemoc nebyl zatím objeven, klíčovým krokem je nalezení molekulárních biomarkerů, které by pomohly nemoc předvídat či detekovat ji v prvopočátcích. V dnešní době jsou intenzivně studovány biomarkery z řad lipidů, jelikož nemocí zasažený orgán, mozek, je z velké většiny tvořen právě jimi. Jako slibné biomarkery Alzheimerovy nemoci se v několika studiích ukázaly být fosfatidylcholiny, které prokázaly změněné hladiny u pacientů trpících touto chorobou, ve srovnání se zdravými lidmi. S využitím lipidomického přístupu analýzy, vybrané fosfatidylcholiny byly extrahovány z lidské plazmy, separovány pomocí vysokoúčinné kapalinové chromatografie, detekovány pomocí hmotnostní spektrometrie a kvantifikovány metodou standardního přídavku. Během analýzy byla testována různá extrakční činidla, chromatografické kolony, mobilní fáze a gradienty, a fosfatidylcholiny byly detekovány několika hmotnostními spektrometry. Finální kvantifikace vybraných fosfatidylcholinů v lidské plazmě byla provedena pomocí vysokoúčinné kapalinové chromatografie ve spojení s kvadrupólovou iontovou pastí, přičemž ze získaných dat byly navíc vypočítány návratnosti extrakcí, matricový efekt plasmy a limity detekce a kvantifikace.

# Summary

Alzheimer's disease is a serious neurodegenerative disease, having characteristics of memory losses and inability to carry out everyday activities. Although, the cure for this disease has not been discovered, it is crucial to find molecular biomarkers that would help the prediction or early detection of the disease. Nowadays, intensive search for the biomarkers is focused in the group of lipids, since the affected organ, brain, is mostly composed from those. As promising biomarkers of Alzheimer's disease, phosphatidylcholines were proposed in several studies, since they showed altered levels in patients suffering from this disease, in comparison to healthy people. Using lipidomics approach in our study, chosen lipids were extracted from human plasma, separated by high performance liquid chromatography, detected by mass spectrometry and quantified by standard addition method. Different extraction solvents, chromatographic columns, mobile phases and gradients were tested, in addition to detection of phosphatidylcholines by several mass spectrometric techniques. The final quantification of phosphatidylcholines in human plasma was obtained by high performance liquid chromatography coupled to quadrupole ion trap, while the extraction recoveries, matrix effect of plasma and limits of detection were calculated from the acquired data.

## Acknowledgement

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# Content

# AIMS OF THE STUDY

- Literature summary of topics: lipidomics, lipids, lipid analysis, high performance liquid chromatography, mass spectrometry, lipid quantification, Alzheimer's disease
- Development of suitable LC-MS method for analysis of phosphatidylcholines
- LC-MS analysis of chosen phosphatidylcholines by different mass spectrometric techniques
- Quantification of chosen phosphatidylcholines in human plasma

# INTRODUCTION

Alzheimer's disease is a serious neurodegenerative disease that affects more than 35 million people worldwide (World Health Organization, 2012). Characteristic features for the disease are accumulation of amyloid plaques and neurofibrillary tangles in brain, leading to loss of neurons and synapses (Hooijmans *et* Kiliaan, 2008). The alternations in brain are reflected by changes in behaviour, memory losses, confusion and in later phase by inability of self-care and finally death (Burns *et* Illife, 2009). Since the brain is mostly formed by lipids, the attention is focused on lipid research nowadays. The new science studying lipids is called lipidomics and it represents one of the branches of metabolomics, characteristic by study of small metabolites under 1500 Da (Baker, 2011; D'Alessandro *et* Zolla, 2012; Navas-Iglesias *et al*., 2009). Lipidomic analysis is typically characterized by several steps – extraction of lipids from the biological sample, their separation, detection and finally identification and quantification (Carrasco-Pancorbo *et al*., 2009). The fact complicating lipid research is incredible chemical diversity of this group. The main technique used in lipidomics is mass spectrometry, which detects molecules based on their mass to charge ratio (Kofeler *et al*., 2012). The mass spectrometry (often coupled with high performance liquid chromatography) is efficient tool for lipid analysis, however it is also limited by the existence of high number of different lipid isobars and isomers, characterized by the same molecular mass (Ekroos, 2012). Therefore, for reliable identification and quantification of lipids, it is important to introduce also other techniques, such as nuclear magnetic resonance or special types of chromatography (Cajka *et* Fiehn, 2014; Carrasco-Pancorbo *et al*., 2009). As regards Alzheimer's disease, one of the main goals of lipidomics is to find biomarkers, which would help the prediction or early detection of this disease. It has been already shown, that lipids from the class of phosphatidylcholines, as the main components of biological membrane, could be potential molecules with biomarker function and it would be useful to provide further research of those (Mapstone *et al*., 2014; Oresic *et al*., 2011; Whiley *et al*., 2014).

# LITERATURE SUMMARY

## 1 Lipidomics

As an emerging discipline, systems biology places emphasis on the relationship between cellular components as they interact to form whole systems. The study of these components and their relevant systems can be broken down into four main categories, or "omics sciences" – genomics, proteomics, transcriptomics and metabolomics (Figure 1; Wenk, 2005). The sub-field of metabolomics studies small compounds (generally under 1500 Da) involved in metabolism within a cell - nucleotides, sugars, amino acids and lipids. These molecules (metabolites) are referred to as metabolome in their entirety (Baker, 2011; D'Alessandro *et* Zolla, 2012). Since there is incredible chemical diversity within the metabolome, it led over time to the cleavage and formation of new scientific branches, namely glycomics, studying sugars, and lipidomics, analysing lipids (Navas-Iglesias *et al*., 2009).

Lipidomics can be simply defined as the scientific study of lipidome – the collection of all existing lipids within a cell. More specifically, it examines the function of lipids, their metabolism, relationships and interactions with other molecules (Navas-Iglesias *et al*., 2009). Lipidomics seeks to identify all lipid species and characterize their relationships with proteins related to lipid metabolism and function, with regard to importance of gene expression and regulation (Spener *et al*., 2003).

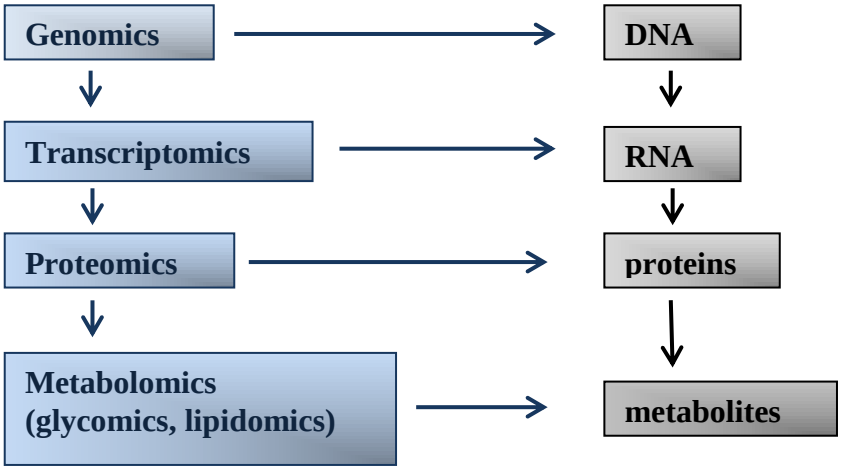


Figure 1: "Omics" sciences (created by Navas-Iglesias *et al*., 2009; Zhang *et al*., 2010)

## 1.1 Targeted, untargeted versus focused lipidomics

Lipidomics and metabolomics have two basic approaches to the research of small molecules – targeted and untargeted. Since the border between targeted and untargeted lipidomics is very often unclear, third field was created – focused lipidomics. At present, the analysis of lipids is performed primarily using mass spectrometry (MS), and therefore the lipidomics classification is based on the type MS analysis used (Navas-Iglesias *et al*., 2009).

According to this classification, targeted lipidomics involves selecting specific lipids and quantifying them within a sample. In targeted analysis, there is need for prior knowledge of the structure of the molecule being studied, its exact mass and how it behaves during the fragmentation step. Therefore, in MS analysis, it is typical to use specific scans, such as multiple reaction monitoring (MRM) (Navas-Iglesias *et al*., 2009). An example of the targeted lipidomics approach is the study of individual phosphatidylcholine (PC) species with known exact masses and fragmentation profiles, as will be shown in our experiment.

In contrast, untargeted lipidomics searches for all lipids present in the sample using high-resolution MS instruments (Orbitrap, quadrupole-time-of-flight MS). The unknown exact masses are then compared with available databases in order to identify them. From targeted to untargeted lipidomics, the number of analysed molecules increases and the sensitivity of detection for less abundant molecules decreases (due to chosen MS techniques) (Navas-Iglesias *et al*., 2009).

Focused lipidomics lies between these two approaches, and it is oriented towards a chosen group of lipids. While there is a need to have certain information about the lipids being studied, it is not necessary to know their exact masses or the whole of their fragmentation behaviour. Studied molecules are analysed by MS scans such as the precursor ion scan, product ion scan and neutral loss scan. An example of focused lipidomics is the use of positive ionization mode to study of lipids from the phosphatidylcholine group, using the knowledge that the fragmentation ion of *m/z* 184 Da belonging to the phosphocholine "head" is common to all members of PC family (Taguchi *et al*., 2005).

## 1.2 LC-MS versus shotgun lipidomics

Lipidomics can be further divided by use of different types of analysis in two main parts – LC-MS lipidomics and shotgun lipidomics. LC-MS lipidomics is characterized by separation of lipid species by liquid chromatography (LC) followed by detection of these lipids using mass spectrometry. This approach is more time consuming, however, its advantage is reliable identification and detection of less abundant lipid species with increased sensitivity. Furthermore, LC-MS analysis using MRM scan mode is a common option for lipid quantification (Cajka *et* Fiehn, 2014; Ekroos, 2012; Han *et* Gross, 2005).

Shotgun lipidomics is performed using mass spectrometry without the LC separation step. The lipid mixture is injected directly into an ion source (generally electrospray) and is analysed using different types of MS scans. The advantage of shotgun lipidomics is shorter time of analysis, constant ion suppression effect and the possibility of intra-source molecule separation (will be discussed later in Chapter 5.4 Mass spectrometry of lipids) (Ekroos, 2012; Han *et* Gross, 2005).

# 2 Lipids

Lipids are group of natural substances that are present in every living cell and organism. Earlier, they were characterized as chemicals insoluble in water and soluble in organic solvents, however this characteristic is not common to all lipids (Smith, 2000). Presently, according to the new nomenclature created by Lipid Maps Consortium, lipids are defined as hydrophobic or amphipathic molecules that are created by two synthetic ways - carbanion based condensation of thioesters or/and by carbocation based condensation of isoprene units (Fahy *et al.*, 2005). Other authors define lipids as fatty acids, their derivatives and the compounds that are functionally or biosynthetically connected to them. In general, the lipid class includes structurally different compounds such as fatty acids, fats, sterols, waxes, phospholipids, eicosanoids, fat soluble vitamins, terpens, prenols and others (Christie *et* Han, 2012).

## 2.1 Function of lipids

Lipids are biologically active compounds and have several important functions. Some serve as energy sources, some play critical roles in cell signalization, and others form cell membranes.

### 2.1.1 Lipids as an energetic source

Lipids in the form of triacylglycerols are an ideal source of energy. Compared with proteins and sugars, they are stored in a less oxidized and anhydride form, making their oxidation per unit weight more efficient. Triacylglycerols (TAG, Figure 2) taken from food are first emulsified by bile acids, then digested by lipases to glycerol and fatty acids and absorbed to intestine lumen. In intestinal cells, they are re-esterified back to TAG and with phospholipids, cholesterol and apolipoprotein are incorporated in a lipoprotein complex called chylomicron. These lipoprotein complexes are transferred through the lymph system to blood and then towards tissues. In times of need, triacylglycerols are hydrolysed by lipoprotein lipases and the fatty acids released in this process are taken up by cells. Fatty acids can be either re-esterified back to TAG and stored in adipose tissue, or oxidized in a process called β-oxidation occurring in the matrices of mitochondria in muscle cells. During β-oxidation, fatty acid molecules are shortened in every round by two carbons, creating molecules of acetyl coenzyme A. Acetyl-CoA is further metabolized in the citric cycle, generating reduced coenzymes NADH+H+ and $FADH_2$. β-oxidation yields these coenzymes as well, and all are finally oxidized in an electron transport chain resulting in ATP formation (Voet *et* Voet, 2011).

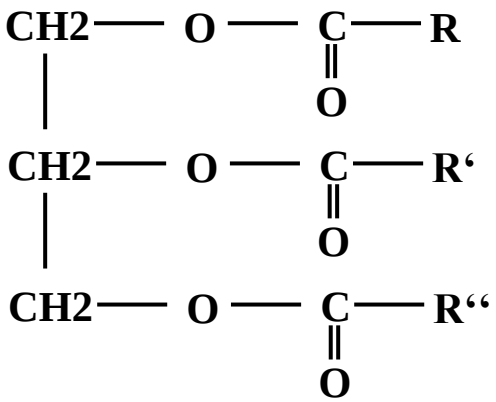


Figure 2: Scheme of triacylglycerol where R, R’ and R’’ are fatty acyls (created by http://www.google.com/patents, 2015)

### 2.1.2 Lipids as signalling molecules

Lipids can also act as signalling molecules. A typical example of a lipid signalization pathway is shown in Figure 3. There are two second messengers - diacylglycerol (DAG) and inositol-3-phosphate (IP3) which are created from phosphatidylinositol-4,5-biphosphate (PIP2). The hydrolysis of PIP2 is secured by enzyme phospholipase C (PLC), which is first activated by the α subunit of G-protein (G-α), which is released from the G protein-coupled receptor (GPCR). IP3 is then released from the membrane and acts as ligand for calcium channels in the smooth endoplasmic reticulum. This results in a release of calcium ions into the cytosol where they react with enzyme protein kinase C (PKC), which is transported to DAG anchored in the cytoplasmic membrane. Creation of this complex causes activation of PKC, which subsequently influences activity of other molecules through phosphorylation (Alberts *et al*., 2002).

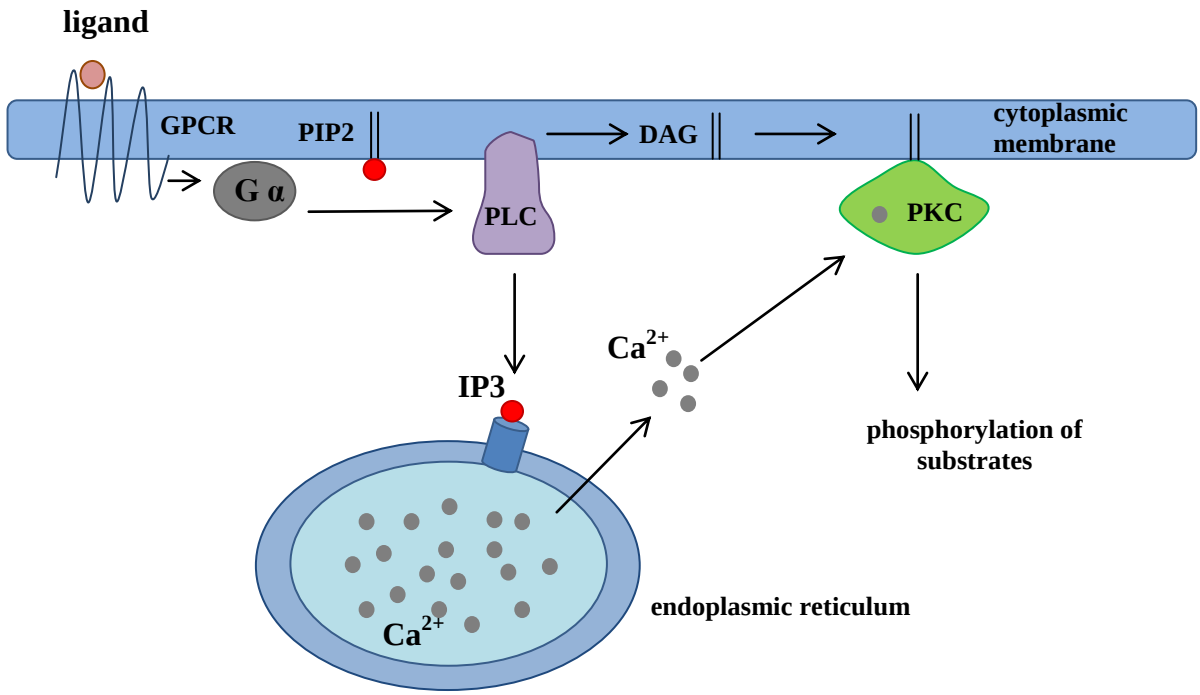


Figure 3: Phospholipid signalization pathway (created by http://en.wikipedia.org; http://courses.washington.edu, 2015)

### 2.1.3 Lipids as membrane structures

Phospholipidic membranes are of critical importance to cell structure and function. The main phospholipidic bilayer - cytoplasmic membrane ~ about 5 nm thick - protects the cell against external influences, separates the inner environment of the cell from the outside, facilitates transportation of chosen molecules and enables cell signalization. In addition to the cytoplasmic membrane, cells contain other lipid membranes that form envelopes of organelles, separating them from one another and from the cytosolic matrix of the cell (Alberts *et al*., 2002; Voet *et* Voet, 2011).

Cytoplasmic membrane consist of 50 % lipids by mass, where approximately half of the lipids are represented by phospholipids (phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylethanolamine) and lipid molecules - cholesterol, sphingolipids and glycolipids make up the remainder. Phospholipids form the foundation of the lipid bilayer, as their nonpolar, hydrophobic fatty acid tails turn to produce the inner membrane, and their polar heads (consisting of phosphocholine, phosphoethanolamine, etc.) face the aqueous external environment on either side (Figure 4) (Alberts *et al*., 2002).

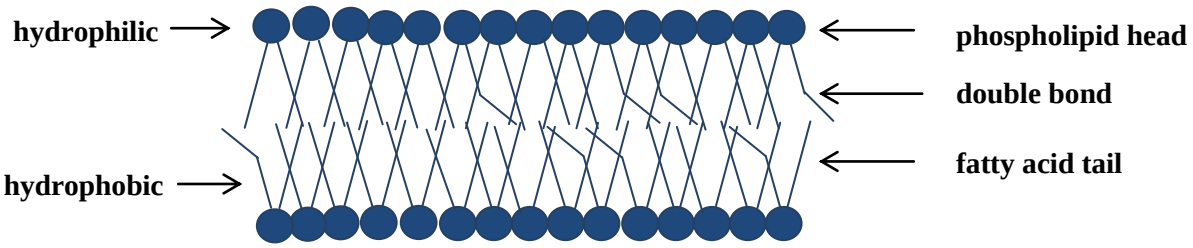


Figure 4: Schematic picture of phospholipidic bilayer (created by http://medicine-science-and-more.com, 2015)

The fatty acids in phospholipids can be saturated (without double bonds) or unsaturated (with one or more double bonds), with the kinked tails of unsaturated fatty acids serving to loosen the membrane, and thereby enhancing membrane fluidity. Another important molecule influencing membrane fluidity is cholesterol, which, while making the membrane less fluid, increases molecular distances and protects membrane against crystallization. Because of membrane fluidity, lipids can quite freely move in a lateral direction (on one side of the leaflet), and rotate around their axis. In contrast, “flip flop”

or transverse diffusion from one leaflet to the other is very rare. An important characteristic of the cell membrane is also its asymmetry – sphingomyelins and phosphatidylcholines are mostly present in the outer leaflet, while phosphatidylethanolamines and phosphatidylserines are located in the inner leaflet. Glycolipids, glycoproteins and proteoglycans form the saccharide envelope of the cell (glycocalyx), which serves to protect the cell and provide specific sites for cell recognition, which are located exclusively in the outer leaflet (Alberts *et al*., 2002; Rothman *et* Lenard; 1977; Voet *et* Voet, 2011).

The second half of the cytoplasmic membrane's mass is comprised of proteins. According to the fluid mosaic model (Singer *et* Nicolson, 1972), protein molecules sail in phospholipidic bilayer as "boats in the sea". They permit the transport of large molecules through the membrane, play a role in cell signalization and can act as enzymes or receptors. All membranes are naturally semipermeable, meaning that they are only able to be permeated by nonpolar (e.g. $CO_2$) and uncharged polar compounds (e.g. $H_2O$) while other molecules (polar, e.g. glucose or charged, e.g. $K^+$, $Na^+$) have to be transported through specialized protein pumps, transporters and channels. Transport enabled by protein transporters and pumps is largely dependent on energy, and therefore referred to as "active"; while diffusion through channels, being entirely energy independent, is called "passive". Diffusion through channels is driven by concentration gradient, electrical potential or a combination of the two (electrochemical potential) (Alberts *et al*., 2002).

## 2.2 Classification of lipids

For a long time, lipids were divided into separate categories based on function, structure or other properties, with no universally accepted classification system. In 2005, LIPID Metabolites and Pathways Strategy Consortium (LIPID MAPS; http://www.lipidmaps.org) created a comprehensive lipid classification system, dividing lipids in categories according their structure and mechanism of chain extension. This system yielded eight main groups - fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), saccharolipids (SL), polyketides (PK), sterol lipids (ST) and prenol lipids (PR). Lipids from categories FA, GL, GP, SP, SL and PK arise from ketoacyl chain extension while lipids from categories ST and PR are synthetized by isoprene chain extension (Fahy *et al*. 2005).

- Fatty acyls

The group of fatty acyls is one of the most important lipid categories since the main structure - fatty acyl - forms the building block of complex lipids. The fatty acyl group contains 14 classes (fatty acids and conjugates, eicosanoids, fatty esters, fatty amides, etc.) with eicosanoids (prostaglandins, leukotrienes and thromboxanes), playing an integral role in inflammation and immunity (de Caterina *et* Basta, 2001; Fahy *et al*., 2005; http://www.lipidmaps.org, 2015).

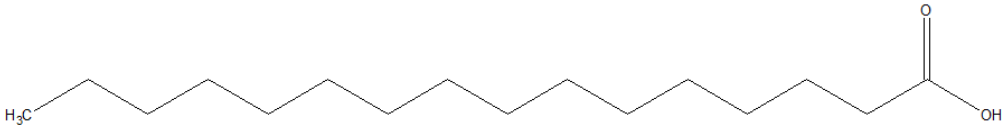


Figure 5: Palmitic acid (C16:0) from group of fatty acyls

- Glycerolipids

Glycerolipids are represented by six classes (e.g. monoradylglycerols, diradylglycerols, glycosylmonoradylglycerols, where radyl means acyl, alkyl or 1-alkenyl substituent - http://www.chem.qmul.ac.uk, 2015). The main functions of glycerolipids are energy storage (triacylglycerols) and cell signalization (diacylglycerols) (Alberts *et al*., 2002; http://www.lipidmaps.org, 2015).

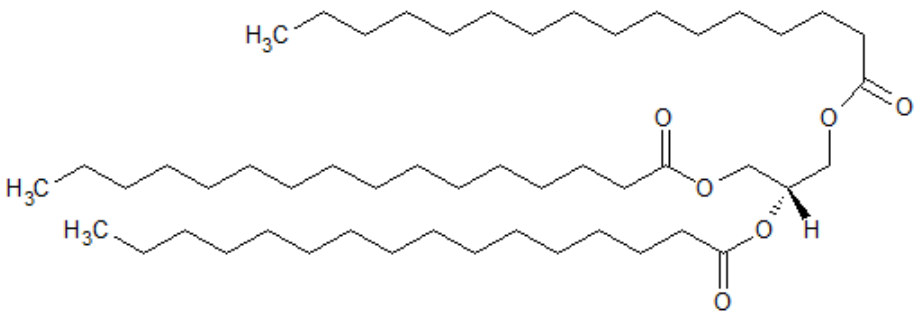


Figure 6: TG (16:0/16:0/16:0) from group of glycerolipids

- Sphingolipids

Sphingolipids are lipids containing a sphingoid base backbone. They consist of ten classes (ceramides, sphingoid bases, neutral glycosphingolipids, etc.) and are extremely

active in cell membranes (signalization, cell-to-cell interaction, recognition) (Bartke *et* Hannun, 2009; Fahy *et al*., 2005; http://www.lipidmaps.org, 2015).

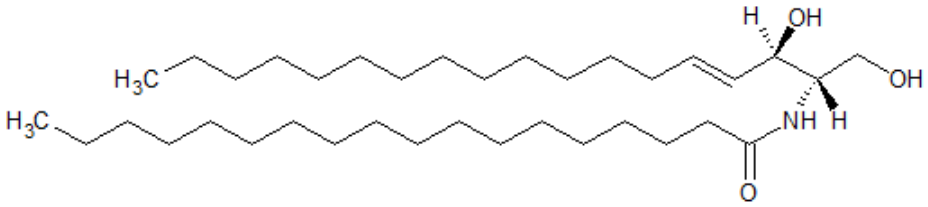


Figure 8: Cer (d18:1/18:0) from group of ceramides

- Saccharolipids

Saccharolipids are a category of lipids where fatty acids are bound directly to a sugar. They are divided into six classes (acylaminosugars, acyltrehaloses, acyltrehalose glycans, etc.) and can, for example, act as precursors to lipopolysaccharides in the outer leaflet of some bacterial cell membranes (Fahy *et al*., 2005, http://www.lipidmaps.org, 2015).

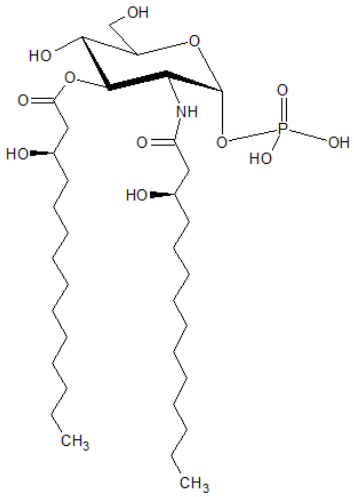


Figure 9: Lipid X from the group of saccharolipids

- Polyketides

This lipid group can be divided into 15 classes (linear polyketides, polyenes, linear tetracyclines, flavonoids etc.) and includes molecules that may be used as antimicrobial and anticancer drugs (however they can also act as potential toxins) (Fahy *et al*., 2005; http://www.lipidmaps.org, 2015).

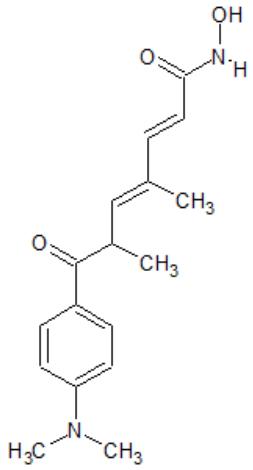


Figure 10: Trichostatin from the group of polyketides

- Sterol lipids

Sterol lipids have six classes (sterols, steroids, steroid conjugates, etc.) and include one of the most common natural lipids, cholesterol. Sterol lipids contain many molecules with important biological functions, e.g. steroid hormones (progesterons, estrogens, androgens, mineralocorticoids and glucocorticoids), vitamin D or bile acids (http://www.lipidmaps.org, 2015).

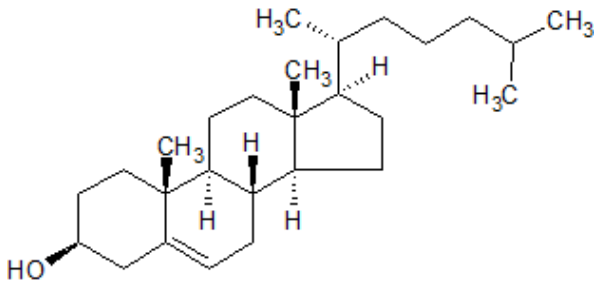


Figure 11: Cholesterol from the group of sterols

- Prenol lipids

There are five classes of prenols (isoprenoids, polyprenols, hopanoids, etc.) and their basic segment, isoprenoid, is created by condensation of dimethylallyl diphosphate and isopentenyl diphosphate. The isoprenoid carotenoid functions as a natural anti-oxidant as well as quinones involved in cellular respiration (Alberts *et al*., 2002; Fahy *et al*., 2005; http://www.lipidmaps.org, 2015).

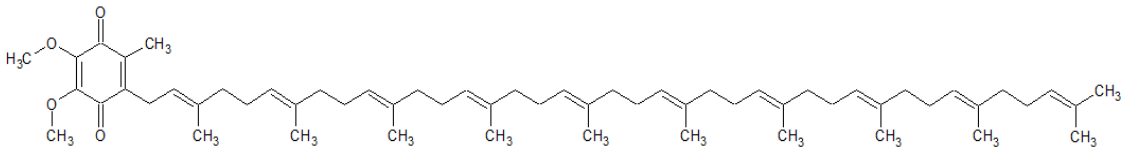

Figure 12: Coenzyme Q10 from the group of prenols

- Glycerophospholipids

Glycerophospholipids are a type of polar lipid, meaning they contain hydrophilic and hydrophobic parts. The hydrophobic „tail“ is formed from two fatty acid chains, and the hydrophilic „head“ may be composed of a number of different compounds. The compounds making up the head are the criterion by which glycerophospholipids are divided into six main classes – glycerophosphocholines, glycerophosphoethanolamines, glycerophosphoserines, glycerophosphoglycerols, glycerophosphates and glycerophosphoinositols. The most important function of phospholipids is their role in composing cellular membranes (see Chapter 2.1.3 - Lipids as membrane structures) (Alberts *et al.*, 2002; Fahy *et al.*, 2005; http://www.lipidmaps.org, 2015).

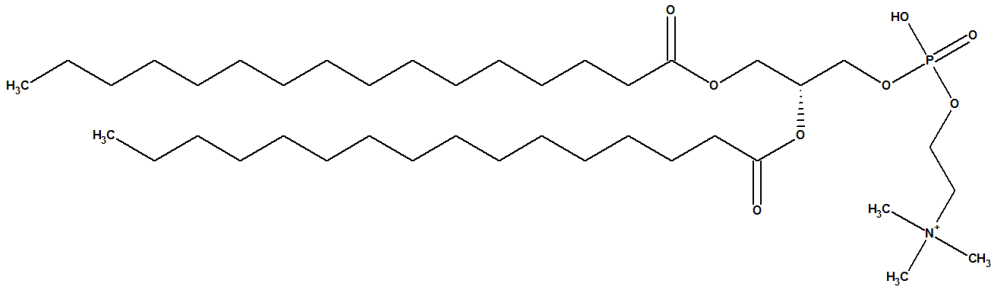

Figure 13: PC (16:0/16:0) from group of glycerophospholipids

## 2.3 Phosphatidylcholines

Phosphatidylcholines, or more accurately diacylglycerophosphocholines (PCs), are one of the nine subclasses of glycerophosphocholines. Their general structure is represented in Figure 14. The molecule is fabricated from a glycerol backbone, which is esterified at the *sn*-1 and *sn*-2 position by fatty acyls and at the *sn*-3 position by phosphocholine (*sn* - stereospecific numbering - http://www.chem.qmul.ac.uk, 2015) (Watson, 2006; http://www.lipidmaps.org, 2015).

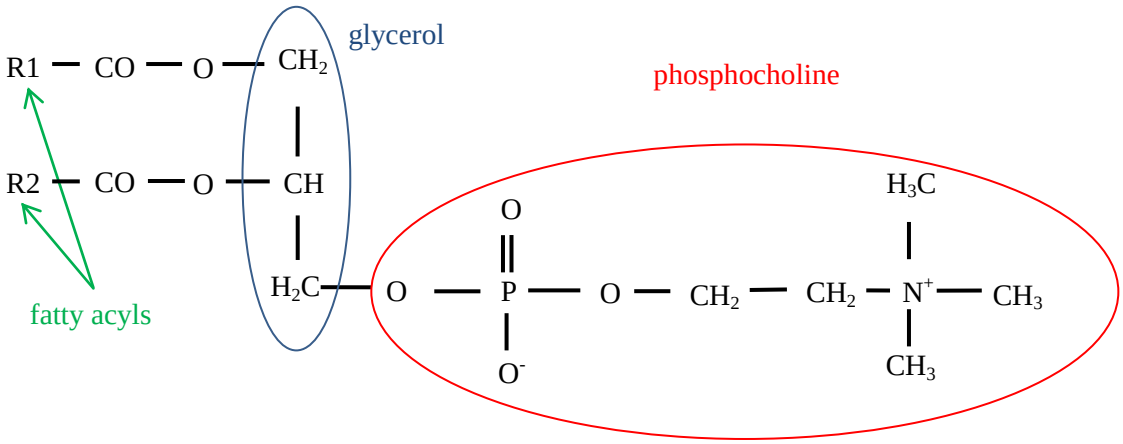


Figure 14: Phosphatidylcholine scheme (created by http://nootropicsupplementreview.com, 2015)

Chemically, phosphocholine head is zwitterionic, meaning it contains both positive and negative charge, which leads to no total net charge and whole PC molecule neutrality. Fatty acids which make up the hydrophobic tail contain between 14 to 22 carbons (mostly even numbers) and can be saturated or unsaturated. Unsaturated fatty acyls are in most cases present at the *sn*-2 position while saturated fatty acyls tend to be present at the *sn*-1 position. The number of double bonds can be between one and six, and may be, in theory, positioned anywhere within the fatty acid molecule. However, most double bonds are located in the *cis* position from the carboxylic group. Because of different chain lengths, their positioning on PC molecules (*sn*-1 vs *sn*-2), and the variations in the number and positions of double bonds, the group of PCs exhibits high diversity, which complicates their study (will be discussed later in Chapters 3, 4, 5 and 6) (Ekroos, 2012; Christie *et* Han, 2012; http://lipidlibrary.aocs.org, 2015).

Phosphatidylcholines have several important functions: they can be found in the cytoplasmic membrane where they represent about 50 % of all phospholipids, they are part of lipoprotein molecules (PC is the most abundant phospholipid in plasma) and they act as biochemical precursors of signalling molecules such as sphingomyelin, lysophosphatidylcholine and platelet-activating factors (Gibellini *et* Smith, 2010; http://lipidlibrary.aocs.org, 2015).

# 3 Lipid analysis

Lipids, as naturally present biological compounds, can be analysed using different scientific approaches. The traditional method of lipid analysis is presented in the Figure 15. The first step is the extraction of lipids from the tissue or biological solution, followed by lipid separation, which may be performed using a variety of techniques. Specialized detectors distinguish separated lipids and finally, the data is collected and analysed using specialized software designed for lipid identification and quantification (Carrasco-Pancorbo *et al.*, 2009).

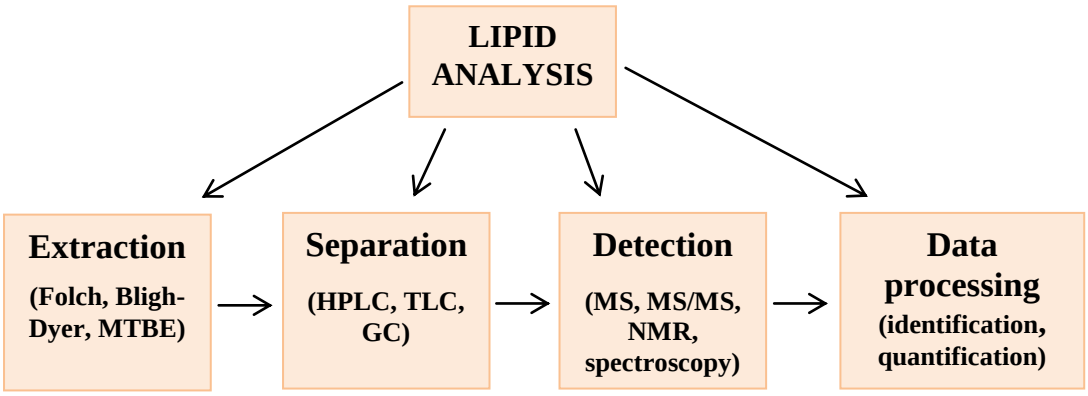


Figure 15: Lipid analysis scheme (Cajka *et* Fiehn, 2014; Carrasco-Pancorbo *et al.*, 2009)

## 3.1 Extraction of lipids

The first step of lipid analysis is lipid extraction. Extraction is meant to separate chosen lipids from the sample matrix and remove other molecules (e.g. proteins) prior the analysis. Lipids can be extracted from different biological samples – animal or plant tissues, bacterial and cell cultures, plasma or blood samples, urine etc. The most efficient extraction method is chosen based on the type of sample and class of lipids being analysed. However, given the incredible chemical diversity of the lipid group, there is no extraction procedure leading to the acquisition of all lipids during one analysis. Using the simple "*like dissolves like*" rule, the nonpolar lipids (triacylglycerols) are efficiently extracted by less polar solvents (hexane), and polar lipids (phospholipids) by more polar solvents (methanol and water) (Cajka *et* Fiehn, 2014; Carrasco-Pancorbo *et al.*, 2009; Christie *et* Han, 2012).

The most common extraction methods are the Folch method and its modification, the Bligh-Dyer method, both of which are based on the use of a chloroform-methanol solution. The Folch method (Folch *et al*., 1957) uses a two-step extraction by chloroform-methanol-water in ratio 8:4:3 by volume. In the first step, chloroform-methanol in ratio 2:1 is added in 20 times volume of the tissue or solution, and in the second step, the mixture is washed by water by 20% of its volume. Extracted lipids are located in the lower organic phase (with the composition of solution - chloroform-methanol-water in ratio 86:14:1) while the upper phase (chloroform-methanol-water - 3:48:47) contains non-lipid molecules. The Bligh-Dyer extraction (Bligh *et* Dyer, 1959) is conducted using chloroform-methanol in ratio 1:2, however, an additional 1 volume of chloroform and 1 volume of water are added to the solution. The advantage of Bligh-Dyer method is that it is simple and less time-consuming.

Other extraction techniques are based either on methanol-chloroform extraction with small modifications (changed ratios of chloroform-methanol, additions of particular chemicals), or simple extraction solvents as acetonitrile or methanol can be used, though in those cases, some loss in extraction efficiency is to be expected (Cajka *et* Fiehn, 2014). One of the recently introduced extraction methods was methyl-*tert*-butyl ether extraction (MTBE) (Matyash *et al*., 2008). The advantage of MTBE method is the replacement of chloroform, potential carcinogenetic, with methyl-*tert*-butyl ether, methanol and water (in ratio 10:3:2.5). Also, this method does not contaminate the sample with precipitants, since the organic phase containing the extracted lipids is in the upper layer (Figure 16), and to acquire targeted lipids, it is not necessary to go through unwanted protein-precipitants, as in Folch method.

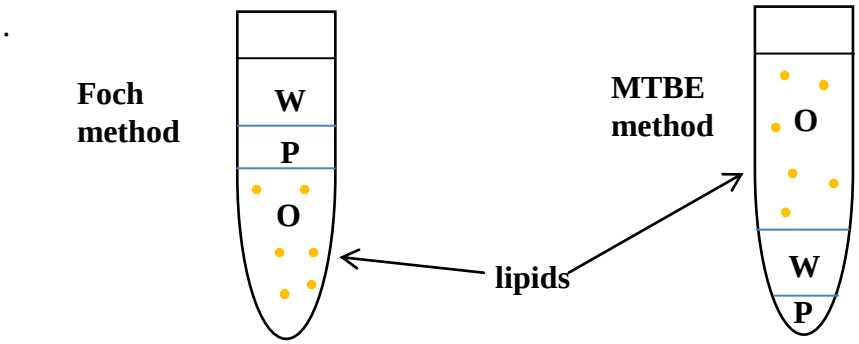


Figure 16: Phase and lipid distribution in Folch and MTBE extraction methods, where O = organic phase, W = water phase, P = precipitant (Matyash *et al.*, 2008)

## 3.2 Separation of lipids

The separation of lipids is an important step helping reliable identification and enabling the detection of less abundant species. However, with the development of efficient mass spectrometry, this step can be omitted (shotgun lipidomics). Lipids can be separated using a number of analytical methods. At the beginning of lipid studies, the widely used separation technique was thin layer chromatography (TLC), which is easy, fast and enables the separation of lipids into basic lipid classes. However, due to the poor resolution and sensitivity of TLC, it is not possible to separate individual lipid species. Later, gas chromatography (GC) began to be used. Unfortunately, GC is limited by the thermal-stability and volatility of analysed molecules, or by the necessity to introduce particular preparation steps, such as derivatization. However, GC is nowadays frequently used tool for nonpolar lipid (TAG) and fatty acid analysis (Cajka *et* Fiehn, 2014; Watson, 2006; Wenk, 2005).

Presently, lipid separation is provided mainly by high performance liquid chromatography (HPLC), which is described in detail in Chapter 4. From the other chromatographic techniques, supercritical fluid chromatography is an expanding field lately. In addition, chiral LC or silver ion chromatography can be used for particular analyses (e.g. for the study of double bond position). It is also possible to separate chosen lipids using electrophoretic techniques such as capillary electrophoresis, however these techniques are not commonly used, as their main requirement is the aqueous solubility of the molecules which is very difficult to provide throughout the lipid group (Cajka *et* Fiehn, 2014; Carrasco-Pancorbo *et al*., 2009).

## 3.3 Detection, identification and quantification of lipids

The next steps of lipid analysis are detection, identification and quantification. There are various types of detectors that analyse chromatographically separated molecules depending on different characteristics. Earlier, detectors measuring refractive index, electrochemical characteristics, spectrophotometric absorbance etc. were used. However, with the development of mass spectrometry, these detection methods have become less common. Mass spectrometry, as the main technique in lipids studies, is further described in Chapter 5. From spectroscopic techniques, nuclear magnetic resonance (NMR) is a useful technique in lipidomics. NMR enables reliable structure identification, as well as molecule quantification;

however the disadvantage of this method is lower sensitivity, which complicates the analysis of less abundant species (Carrasco-Pancorbo *et al*., 2009).

After the detection, acquired information needs to be analysed. Particularly in untargeted lipidomics, a large amount of data is acquired and needs to be processed. The processing steps are represented by normalization and transformation of the data with further spectral smoothing, alignment, peak identification, statistical analysis etc. (Pravdova *et al*., 2002). Software packages enabling lipid data processing include Lipid View, LipidXplorer, Lipid Inspector and others (Kofeler *et al*., 2012). However, the main complication in lipidomics analysis is that a complex database has yet to be developed. In existing databases, such as LIPID MAPS, LIPIDAT, LipidBank, Lipid Library and CyberLipids, one can find information about classification, nomenclature, structure, and biological or physiochemical properties of lipids, but unfortunately, none of these sources provide a comprehensive reference database, as are already existing in genomics and proteomics (Navas-Iglesias *et al*., 2009; Wenk, 2005).

The final, critical step of lipid research, lipid quantification, will be closely described in Chapter 6.

# 4 High performance liquid chromatography

## 4.1 Introduction

High performance liquid chromatography (HPLC) is an analytical method that separates analytes based on their interactions with a stationary and mobile phase. The stationary phase is provided by a column filled with silica particles (often with attached alkyl groups), while certain solvents represent the mobile phase. HPLC can be performed in normal or reversed phase. Normal-phase HPLC (NP-LC) is characterised by a polar stationary phase and nonpolar mobile phase. In contrast, reversed-phase HPLC (RP-LC) is determined by a nonpolar stationary phase and a more polar mobile phase. Recently, a third chromatographic approach called hydrophilic interaction chromatography (HILIC) was introduced. This technique is based on the interaction of water molecules present in mobile

phase with the stationary phase, while stronger retention of highly hydrophilic (polar) compounds in the column is provided (Snyder *et al.*, 2010).

Every HPLC instrument is composed of several main components – mobile phase reservoir, pumping system, sample injector (autosampler), column and detector (Figure 17). Mobile phase is pumped from the reservoir, while the sample is injected into mobile phase flow. The solution moves through the column, where the analytes interact with the stationary phase and separation takes place. When using NP-LC, the retention of polar analytes in the stationary phase is stronger and they exit the column later, while nonpolar analytes interact with stationary phase less and their retention time is shorter. In reversed-phase mode, the elution order is opposite (Snyder *et al.*, 2010).

Two types of elution can be used in chromatography - isocratic and gradient. In isocratic elution, the separation conditions do not change during analysis – meaning the composition of mobile phase does not change over time, and conversely, gradient elution is characterized by the change of mobile phase composition during analysis time. Separated molecules are then detected by UV, refractive index or evaporative light-scattering detectors; or nowadays mainly by mass spectrometers (Christie *et* Han, 2012; Snyder *et al.*, 2010).

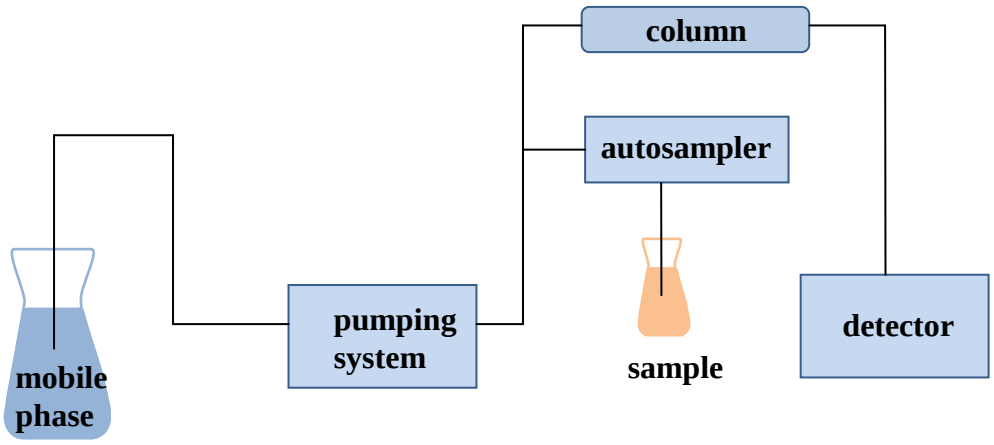


Figure 17: HPLC scheme (created by http://www.waters.com, 2015)

## 4.2 Lipid separation

Lipids can be separated into classes by the chemistry of their polar heads, or into particular species based on their fatty acyl chains. For separation of lipids into classes, NP-LC and HILIC are used, while for analysis of particular features, RP-LC is the method of choice

(Lesnefsky *et al*., 2000; Malavolta *et al*., 2004; McHowat *et al*., 1997). In RP-LC, the retention time of particular lipids in a chosen class is dependent on the length and saturation of the chain. Lipids with longer fatty acyl chains are retained with greater force than the ones having shorter chains and lipids with fewer double bonds have longer retention times, than those with more double bonds (Watson, 2006). In NP-LC, the order of eluted lipid classes is dependent on the composition of mobile phases, meaning when using one mobile phase, the phosphatidylcholines can exit the column before phosphatidylethanolamines, while with the other mobile phase, the phosphatidylethanolamines will exit the column earlier (Christie *et* Han, 2012).

Nowadays, RP-LC with nonpolar stationary phase and polar mobile phase is the most common method for lipid analysis. Stationary phase is mostly represented by 50-150 mm columns with silica particles (~2.5-5 μm), covered preferably by C18 alkyl groups. However, columns packed with particles under 2 μm and thus producing very high pressures and needing special instrumentations (UHPLC – ultrahigh performance liquid chromatography) are coming more commonly used. Mobile phases used in RP-LC are two miscible solvents – an aqueous and organic phase. Aqueous phase is represented by water, often with specific additives improving the shape of chromatographic peaks (e.g. ammonium acetate, formic acid, acetic acid) and as organic solvents, acetonitrile, methanol or isopropanol are widely used (Cajka *et* Fiehn, 2014; Carrasco-Pancorbo *et al*., 2009; Christie *et* Han, 2012).

# 5 Mass Spectrometry

Mass spectrometry (MS) is an analytical technique based on the separation of analytes according to their mass to charge ratio (*m/z*). Every mass spectrometric instrument is composed from three main parts (Figure 18) – ion source, mass analyser and mass detector. The ion source converts molecules into charged and gaseous form, the mass analyser separates ions depending on their *m/z* ratio, and the mass detector transforms the acquired signal into a mass spectrum. The pattern visualized in the mass spectrum is representative of *m/z* ratio relative to ion abundancy (de Hoffman *et* Stroobant, 2007; Ekman *et al*, 2009).

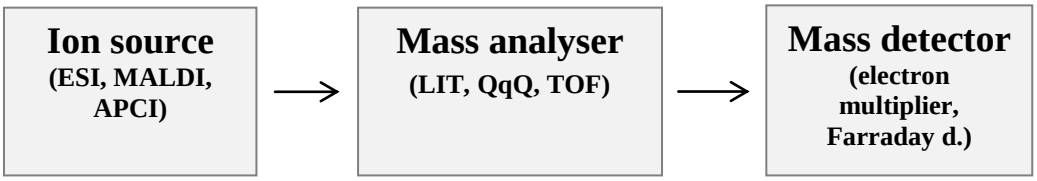


Figure 18: Mass spectrometry scheme (Ekman *et al*., 2009)

## 5.1 Ionization techniques

The ion source, as the first component of mass spectrometer, ionizes molecules and converts them into gas phase. Ionization techniques are divided into two main types – hard and soft. Hard ionization leads to molecule breakage and fragmentation (e.g. electron ionization, chemical ionization), while soft ionization keeps the molecules in an intact state (e.g. ESI - electrospray ionization, MALDI - matrix-assisted laser desorption ionization, APCI - atmospheric pressure chemical ionization) (de Hoffman *et* Stroobant, 2007). The two main techniques used in lipid research are ESI and MALDI (Loizides-Mangold, 2013), although for difficult ionisable molecules (triacylglycerols, sterols), APCI is the preferred method (Byrdwell, 2001).

- Electrospray ionization (ESI)

Electrospray ionization was developed by John Fenn in 1988 (Fenn *et al*., 1989). The general principle of ESI is shown in Figure 19 - the spraying capillary is charged and on its end, an electrical field is formed. A liquid sample is introduced into the nozzle, and thanks to high potential, the liquid gains charge on the surface. At the point when the charge overcomes the surface voltage of the liquid, the liquid changes its shape to a "Taylor cone" (Taylor, 1964). At this point, a spray of droplets occurs, and the ratio of charge to droplet size is increased due to solvent evaporation. The charge of the droplets begin to exceed the physical maximum manageable by surface tension - "Rayleigh limit" (Rayleigh, 1879), leading to a cascade of coulomb explosions characterized by desorption of smaller charged droplets from the surface. The solvent from the droplets is then evaporated using a heated gas or capillary, leading to production of positively or negatively charged gas ions of analyte (de Hoffman *et* Stroobant, 2007; Ekman *et al*., 2009).

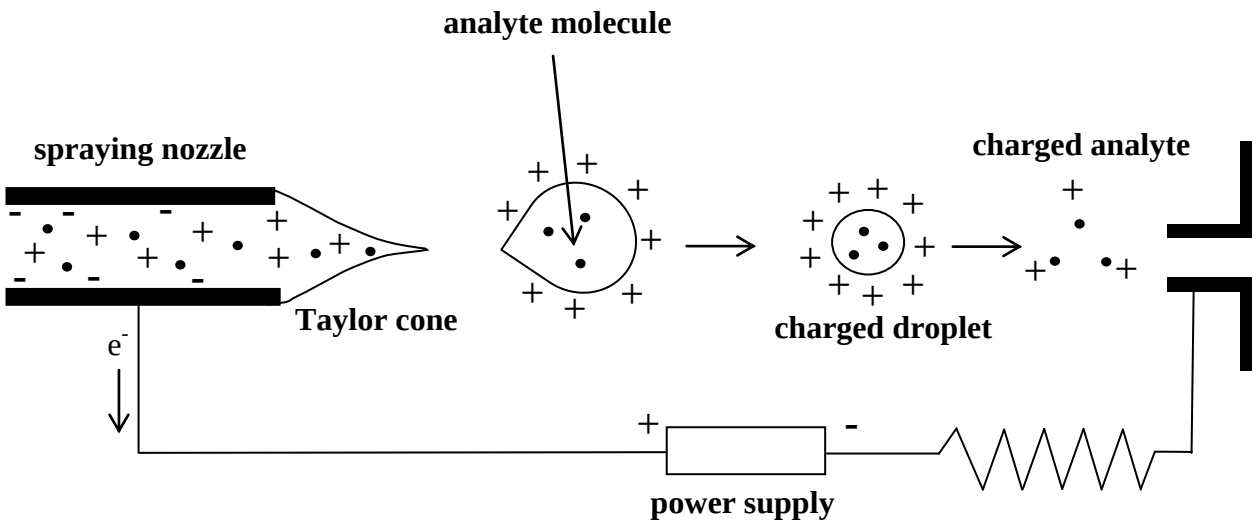


Figure 19: Electrospray ionization scheme (created by http://www.hindawi.com, 2015)

- Matrix-assisted laser desorption ionization (MALDI)

MALDI ionization was first introduced by Franz Karas, Michael Hillenkampf and Koichi Tanaka in the late 1980s (Karas *et al*., 1987; Tanaka *et al*., 1988). It is characterized by the use of lasers to create ions from a biological matrix. First, the sample is mixed with a special matrix composed from a solvent with small organic molecules (for lipids, 2,5-dihydroxybenzoic acid is widely used – Fuchs *et* Schiller, 2009). The solvent is then evaporated and the sample is attacked by laser pulse leading to ablation of the sample layer. In this step, small matrix molecules play an important role – they protect sample molecules and help to absorb laser energy. The matrix is ionized by laser pulse, and the proton is either transferred from the matrix to sample molecules (positive ionization) or is removed from the sample and accepted by the matrix (negative ionization). In conjunction with this ionization, analytes are converted to gas form. The important advantage of MALDI is the possibility of crude sample analysis (e.g. tissue or cell extracts) and relatively high tolerance to sample contamination (de Hoffman *et* Stroobant, 2007; Ekman *et al*., 2009).

One relatively new technique used in lipid research is imaging mass spectrometry, where MALDI ionization takes place. IMS is able to visualize various lipids in thin sections of different tissues (brain, liver tissue, mouse embryo, etc.), allowing for the detection of lipid distributions particular to certain diseases, ontogenesis or other biological processes (Goto-Inoue *et al*., 2011).

## 5.2 Mass analysers

Emerging from the ion source in gaseous phase, ions of the original analyte enter the second part of the mass spectrometer, the mass analyser. Mass analyser separates ions according to their *m/z* ratio while measuring different ion characteristics – velocity of flight, kinetic energy, orbital frequency, etc. Mass analysers widely used for lipidomics analysis include triple quadrupole (QqQ), quadrupole time-of-flight (QTOF), linear ion trap (LTQ), and quadrupole ion trap (Q-Trap) among others (Cajka *et* Fiehn, 2014; de Hoffman *et* Stroobant, 2007).

- Triple quadrupole (QqQ)

Triple quadrupole is a tandem mass spectrometer specified by the connection of two quadrupoles and collision cell placed between them. Every quadrupole is composed from four rods of which two are positively and the other two negatively charged (Figure 20). The rods act as a mass filter where ions are separated using DC (direct current) and RF (radio frequency) voltage, and only ions with a specified *m/z* are able to pass the detector. All other ions come into contact with the charged rods and are subsequently eliminated. In triple quadrupole, the first quadrupole (Q1) separates chosen ions, which are then accelerated towards the second quadrupole. The second quadrupole (q2) utilizes an RF field exclusively for the transmission of the ions to Q3, and uses neutral gas to act as a collision cell, breaking molecules into specific fragments in process known as collision-induced dissociation (CID). Third quadrupole (Q3) then analyses and sorts these fragments prior to their entry into the mass detector. Triple quadrupole is characterized by its lower resolution, but greater scanning speed, enabling the use of specific scan modes suitable for analyte quantification, such as MRM (de Hoffman *et* Stroobant, 2007; Ekman *et al*., 2009).

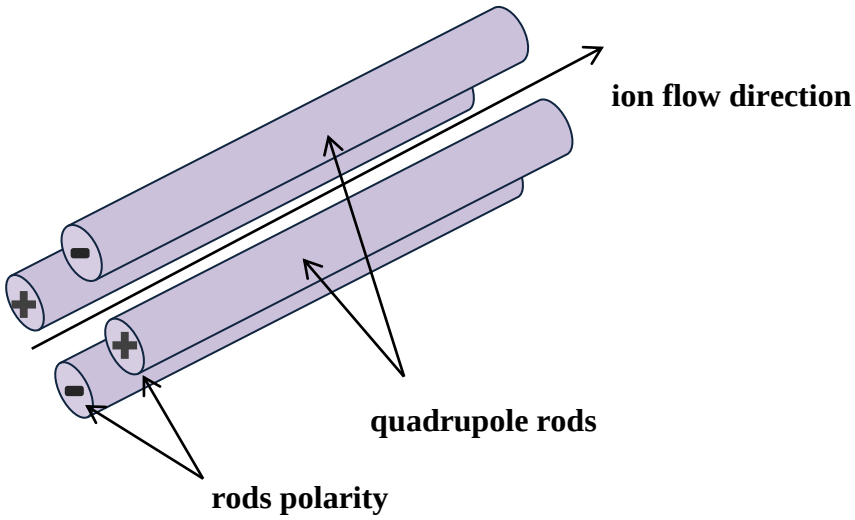


Figure 20: Quadrupole scheme (created by Gross, 2004)

- Linear ion trap (LTQ, LIT)

The linear (or 2D) ion trap is an instrument derived from the quadrupole mass filter. The ions are trapped by an RF field produced by quadrupole rods oriented in radial direction, and by a high potential (DC field) produced by two end electrodes positioned axially. Depending on their *m/z,* ions stored in the trap, pass through the mass detector in single file, and are gradually excited using different RF voltages that correspond with their resonance frequency. Chosen ions kept in the trap may also be fragmented in time by collisions with buffer gas, allowing them to be studied using $MS^n$ analysis. LITs are characterized by increased storage capacity, allowing for greater trapping efficiency compared to a 3D (Paul) trap. Ions stored in the trap can be ejected in two ways, radially through holes in the rods, or axially. Radial ejection is typical for commercial LTQ mass spectrometers, which can exist separately or as a part of Orbitrap mass spectrometers (Douglas *et al*., 2005; Ekman *et al*., 2009; Hopfgartner *et al*., 2004; Thermo Fisher Scientific, 2011; Thermo Scientific, 2009).

Axial ejection of ions is common to another type of mass analyser, the quadrupole ion trap (Q-Trap). The quadrupole ion trap analyser is a hybrid mass spectrometer composed of quadrupoles and a linear ion trap (Figure 21). It functions practically as a triple quadrupole, where the third quadrupole is replaced by a linear ion trap with axial ion ejection. The

advantage of this instrument is its ability to function either as triple quadrupole or ion trap. As such, it can provide scans typical for QqQ (e.g. MRM, PIS) as well as $MS^3$ analysis typical of the LIT (Douglas *et al*., 2005; Hopfgartner *et al*., 2004). In addition, Q-Trap is able to provide specific measuring modes, e.g. $MRM^3$, a combination of MRM and $MS^3$. When, in the first quadrupole, the ion is selected and passes the second quadrupole, where it is broken into fragments, one of which is chosen and continues to the ion trap, where it is trapped, broken down into further fragments, which are scanned and selected. This mode lowers the influence of background noise in the sample and therefore is great tool for compound quantitation (Schreiber *et al*., 2011).

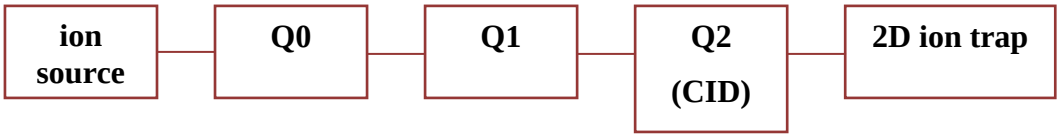


Figure 21: Simplified scheme of Q-Trap (created by http://msr.dom.wustl.edu/, 2015)

- Quadrupole time-of-flight (QTOF)

Quadrupole time-of-flight mass analysers are hybrid mass spectrometers composed from quadrupoles and time-of-flight analysers. The first quadrupole selects ions and is directly connected to second, which cleaves molecules to fragments, leading in to the third part, a time-of-flight analyser. The time-of-flight analyser separates ions depending on their time spent in the apparatus. The lighter ions (with smaller *m/z*) pass the detector faster with the heavier progressing more slowly. Two measuring modes, linear and reflectron, are commonly used with this apparatus. In linear mode, the ions fly directly to the detector while in reflectron mode, the ions are in a set place reflected by a "mirror", allowing them to be registered by the detector. The reflectron mode causes ion flight synchronisation due to the increased length of flight tube, allowing ions with similar *m/z* to pass the detector at the same time, thereby increasing resolution. Consequently, of the advantages of QTOF as compared to QqQ are better resolution and mass accuracy (de Hoffman *et* Stroobant, 2007; Gross, 2004).

## 5.3 Measurement modes in MS/MS

Tandem mass spectrometry (MS/MS) allows fragmentation of ions by collision-induced dissociation (CID) and includes four main scan types - precursor ion scan, product ion scan, neutral loss scan and multiple (selected) reaction monitoring scan (Figure 22) (Griffiths *et* Wang, 2009).

- Precursor ion scan (PIS)

Precursor ion scan is characterized by the selection of a specific product ion produced by CID and scanning for all precursor ions yielding this product ion. The scan allows for the characterization of a chosen group of analytes with similar structure motifs (e.g. all phosphatidylcholines producing 184 Da fragment referring to phosphocholine head in positive ionization mode) (Griffiths *et* Wang, 2009; Taguchi *et al*., 2005).

- Product ion scan

Product ion scans operate via the selection of a specific precursor ion, which is subjected to CID, and following its fragmentation, a scan is run for all of its product ions. This scan allows for the characterization of fragmentation behaviour of chosen molecules (Ekman *et al*., 2009; Griffiths *et* Wang, 2009).

- Neutral loss scan (NLS)

Neutral loss scan is characterized by parallel scanning in the first and third quadrupole, while it is searched for all precursor and product ions giving loss of a defined neutral fragment. NLS and PIS can be used for the identification of groups of molecules with similar fragmentation behaviour (all phosphatidylethanolamines loosing 141 Da neutral fragments belonging to phosphoethanolamine head) (Griffiths *et* Wang, 2009).

- Multiple reaction monitoring (MRM)

MRM is characterized by the selection and fragmentation of a specific precursor ions, and selection of their specific product ions. The primary advantage is increased sensitivity in the detection of chosen ions (Griffiths *et* Wang, 2009).

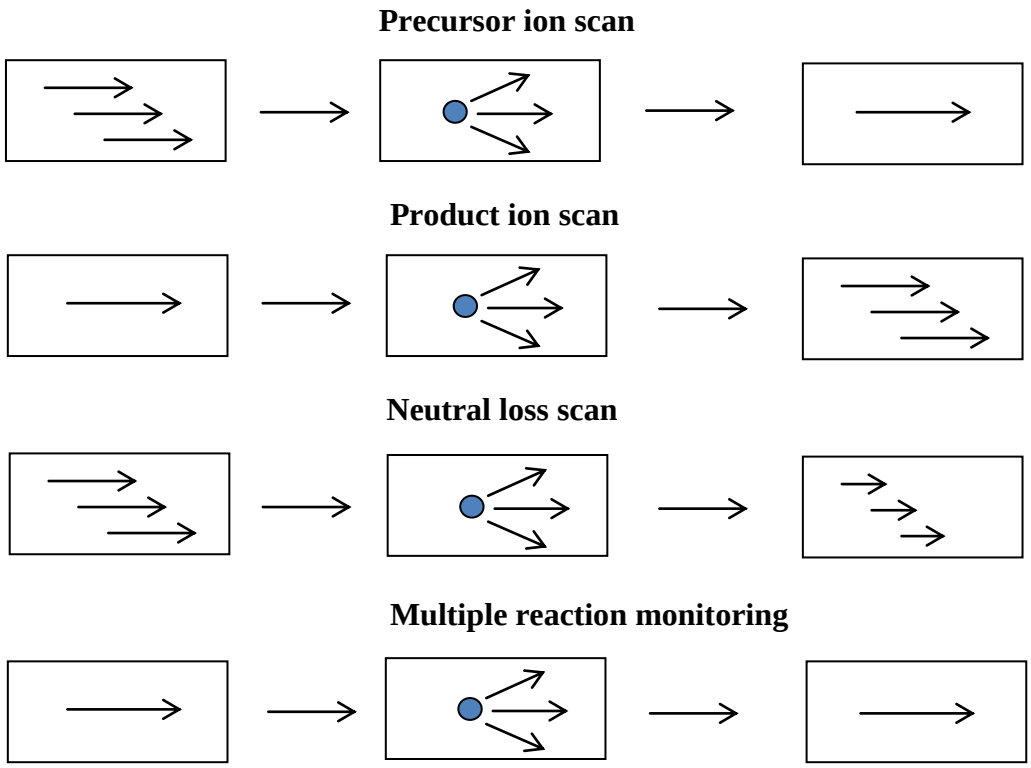


Figure 22: MS/MS scan modes (Griffiths *et* Wang, 2009)

## 5.4 Mass spectrometry of lipids

Mass spectrometry is the most common tool for lipid analysis due to its sensitivity and selectivity. However, as with every scientific method, it has its limitations. The biggest factor complicating reliable lipid identification and quantitation is the enormous chemical diversity of the lipid class. Problems related to MS lipid analysis and some special techniques will be described in this chapter (Kofeler *et al*., 2012).

### 5.4.1 Ion suppression

Ion suppression is a phenomenon occurring in the ion source. The ions compete for the ionization, and the signal of more abundant or better ionisable ions suppresses the signal of less abundant or ionisable ions. In regard to lipids, some of them are naturally abundant and these can cause suppression of less abundant ones which are then difficult to detect. This problem arises especially in shotgun lipidomics, characterized by the introduction of all components to the instrument simultaneously. Suppression effects can be solved by prior

HPLC separation, since the ions are entering the ion source gradually, and less abundant ions may enter the source at different times than the ion suppressors. Suppression effects may also be mitigated by the introduction of a sensitive MRM scan (Carrasco-Pancorbo *et al*., 2009; Ekroos, 2012; Xu *et al*., 2013).

### 5.4.2 Intra-source separation

Although shotgun lipidomics is characterized by no prior chromatographic separation of components in a sample, the separation can occur in the ion source. A new technique called multi-dimensional mass spectrometry-based shotgun lipidomics (MDMS-SL), based on the different ionization properties of different lipid classes, has recently been introduced. Here, lipids are divided in four groups based on their electrical propensities – anionic (e.g. phosphatidylserines), weak anionic (e.g. phosphatidylethanolamines), neutral polar (e.g. phosphatidylcholines) and special lipids (e.g. sterols), and are separated by changing of the pH of solution and using different MS/MS scans. The group of anionic lipids carry negative charge at neutral pH and therefore is very well ionized in negative ionization mode, when using these conditions. If the solution is slightly alkalized by addition of a weak base (LiOH), the second group of weak anionic lipids acquires negative charge and can be well ionized also in negative ESI. Finally, when switching to positive ESI, third group of neutral polar lipids undergoes efficient ionization. Using this knowledge, it is possible to introduce specific MS/MS scans (NLS, PIS) with high accuracy/resolution full scan analyses and acquired data are analysed by a specialized programme enabling identification and quantitation of lipids. The key advantages of this method are the simplicity of sample preparation and analysis, and more importantly, the automation of data processing (Han *et* Gross, 2005; Yang *et* el., 2009).

### 5.4.3 Lipid isobars and isomers

In untargeted lipid analysis, full scan mass spectrums show large numbers of peaks. However, due to the enormous diversity of lipids, different lipids can have similar molecular masses, and one particular lipid with a given molecular mass can exhibit different isobars and positional isomers. Therefore, reliable lipid identification is at times difficult (Ekroos, 2012). Theoretically, it is possible to identify particular lipids using high resolution and high accuracy MS instruments, such as Orbitrap or Fourier transform ion cyclotron resonance. However, for accurate identification, it is necessary to focus on particular classes and exploit

different MS/MS scans (Loizides-Mangold, 2013). Another problem occurs with $C^{13}$ isotopes overlapping, when one peak with a determined molecular mass can be represented by particular lipid species together with an M+2 isotope (with 2 Da higher mass) of another lipid. This problem can be solved through the use of isotope correction software (Han *et* Gross, 2005).

### 5.4.4 Phosphatidylcholine detection

Glycerophosphocholines (PCs) are class of phospholipids consisting of a phosphocholine head and two fatty acyl (FA) chains, a structure which lends itself to great diversity, and therefore, complications with identification. One particular PC with determined mass, e.g. PC (36:2) (36 carbons, 2 double bonds), can exhibit different isobars varying in fatty acyl (FA) chains (PC (18:0/18:2), PC (18:1/18:1), PC (16:0/20:2) and others). Each PC has its positional isomers differing with FA chains at the *sn*-1 or *sn*-2 position (PC (18:0/18:2) vs PC (18:2/18:0)) and each of those can differ with double bond position (10Z, 12Z vs 6Z, 9Z) and double bond stereochemistry (*cis* vs *trans*) (Ekroos, 2012; www.lipidmaps.org, 2015).

For reliable PC identification, it is necessary to use high accuracy instruments, preferably in conjunction with specific MS/MS scans. Using precursor ion scan with product ion 184 Da (phosphocholine fragment) in positive ion mode, it is possible to detect different PC features. From the identified molecular mass of its precursors, one can predict the total number of carbons and double bonds in a PC molecule (e.g. PC (36:1)), however it is impossible to identify a particular PC feature (since there is only one fragment characterizing the whole group). Different fatty acyl chains of PCs can be observed in MS/MS with negative ionization, since fatty acyls are the most abundant fragments (PC (18:0/18:1) will produce two fragments - C18:0 with *m/z* 283.2643 and C18:1 with *m/z* 281.2486) (Ekroos *et al*., 2003).

Positional isomers differing at the *sn*-1 and *sn*-2 position are difficult to identify, since experiments determining FA position from FA ratio in negative MS/MS mode, or by using different collision energy, have historically not been very reproducible (Pulfer *et* Murphy, 2003). For the determination of double bond position, it is necessary to use derivatization leading to adduct production (lipid reactions with lithium, osmium tetroxide, acetonitrile, etc.), or to use special techniques, e.g. ozone-induced dissociation where ozone is delivered with buffer gas directly to the ion trap, causing fragmentation dependent on the position of

double bonds (Ekroos, 2012; Mitchell *et al*., 2009). Another option is the introduction of prior HPLC separation, more specifically, the type called silver-ion chromatography, where silver ions in stationary phase react with double bonds of analytes and enable to separate isomeric lipid features (Carrasco-Pancorbo *et al*., 2009; Holcapek *et al*, 2010).

# 6 Quantification of lipids

The quantification of lipid molecules is the main challenge in lipid research, since it represents the crucial step for discovery of lipid behaviour, and how lipids change in time and during particular biochemical processes or in the presence of diseases. Nowadays, lipid quantification is mainly performed by mass spectrometry. Although the result of mass spectrometric analysis is an abundance of ions (corresponding to the number of detected ions) related to the *m/z*, the number of detected ions does not correspond to the real number of ions present in the sample. It is caused by variable responses from the instrument, which can be influenced by the instrument's settings, the nature of the sample, characteristics of analysed molecules etc. As it concerns lipids, the ionization efficiency varies greatly for different lipid classes, which differ in the composition of lipid heads. The effect of differing acyl chains is minor, however it increases with increasing lipid concentration. Very high lipid concentration also leads to the formation of aggregates, which cannot be properly ionized, preventing a linear response of the instrument and subsequent complication of quantification (Yang *et* Han, 2011).

There are several methods for lipid quantification, and new approaches are still being developed. However, three main methods for molecule quantification are known – external and internal quantification, and the standard addition method. It is also only possible to quantify lipids in a relative way, where one assesses differences between two sets of samples, for example in the study of the differences in lipid profile between healthy and diseased persons (Skoog *et al*., 2013; Yang *et* Han, 2011).

## 6.1 Quantification by external standard

Quantification using external standard is suitable for samples, where no matrix effect is observed. The samples spiked with known concentrations of external standard are prepared

separately in the same way than the unknown samples. Then both sets of samples are measured and the response of the analyte in unknown samples is compared to the responses in the standard samples. Concentration of the analyte in the unknown samples is then calculated. This method confers a disadvantage, that the standard and unknown samples are analysed in different times, which leads to uncertainty concerning the similarity of conditions for both analysed sets. In addition, the procedure of sample preparation can produce changes in sample composition, leading to changes in concentrations which cannot be predicted and corrected (Skoog *et al*., 2013; Yang *et* Han, 2011).

## 6.2 Quantification by standard addition method

Standard addition method is used, when the sample already contains the analyte of interest. This is the case for example when a plasma sample contains specific endogenous compounds, being targeted for quantification. The samples are spiked by the commercial standard of the analyte, either by the same volume of differently concentrated standard, or by different volumes of the same concentrated standard, with aim to acquire different concentrations of the standard in the samples. Then the calibration curve is created and from the acquired linear regression line, the unknown concentration of the molecule in the un-spiked sample (by extrapolation to zero response) is calculated (Figure 23). This approach is rarely used due to its time, sample and chemicals-consuming nature, however, the method is very good for accurate quantification of particular species already present in a sample (Ellison *et* Thompson, 2008; Skoog *et al*., 2013). In regards to phosphatidylcholines, the standard addition method for the quantification of a few compounds can be advantageous, since the ionization efficiency in PC class varies with length and fatty acyl saturation - PCs with shorter and more unsaturated fatty acyl chains are more ionisable, than those with longer, saturated chains (Koivusalo *et al*, 2001).

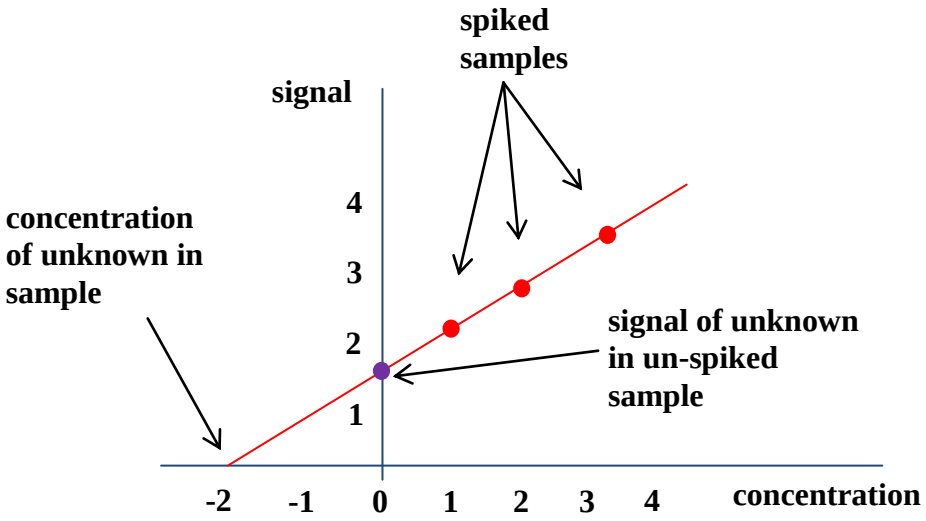


Figure 23: Standard addition method scheme (http://www.tau.ac.il/, http://commons.wikimedia.org/, 2015)

## 6.3 Quantification by internal standard

In lipid studies, quantification using internal standard is a common method. In this application, internal standard is not defined as an isotopically labelled standard of a studied molecule, since few labelled lipids are available and, it is impossible to use labelled standards for all existing lipids. The internal standard, which is used for lipid quantification, has several characteristics – it should be chemically as close as possible to the analyte being quantified, to provide the same analytical behaviour, and it should not co-elute with the analyte during the chromatographic step. The internal standard, compared to the external standard, is typically added prior the sample preparation to undergo all preparations steps. Since the ionization behaviour during the ionization step in mass spectrometry is mostly influenced by the chemistry of the particular lipid class, it is necessary to use at least one analogous internal standard for reliable quantification. This standard cannot be naturally present in the sample, and it is necessary to provide the measurements in low lipid concentrations, as otherwise the effect of different acyl chains starts to prevail (Koivusalo *et al*, 2001; Yang et Han, 2011).

Using the mass spectrometric approach, the quantification of different lipid classes and features can be provided either by LC-MS where measurements in MRM mode are mostly

used, or by shotgun lipidomics with direct infusion of lipids to MS. Shotgun lipidomics quantification approach can be further divided into full scan analyses by high-resolution instruments or in analyses, where specific MS/MS scans are introduced. For many lipidomics analyses, the PIS and/or NLS are used, when at least one internal standard representing a particular lipid class is included. The quantification is then made via the comparison of the response from a differently concentrated internal standard and that of a particular lipid class or species. This approach is suitable for whole lipidomic profiling, however it does not take into account potential lipid isobars, the different ionization efficiency of acyl chains of particular lipids etc. The final step of lipid quantification should be the normalization of the data to some parameter of the sample (content of DNA, protein) and also, the security of $C^{13}$ isotope correction for different lipid isotopologues (Yang *et* Han, 2011, Han *et* Gross, 2005).

One recent approach to untargeted quantification of lipid classes using only one internal standard was introduced by Cifkova *et al*. (2012). It is based on the calculation of response factor characteristic for the whole lipid class and the possibility of its use in future analyses. The simplified scheme of this quantification is shown in the Figure 24. First, the calibration curve of single internal standard is created, then the calibration curve of the lipid standard representing the particular lipid class is provided (lipid standards containing oleoyl acyls were used, since FA 18:1 is one of the most common fatty acids – Holcapek *et al*., 2005), and the response factor of the class is calculated (if response factor of internal standard is equal 1). The unknown concentration of lipid class in the sample is then calculated from the equation in Figure 24, where the concentration of the internal standard, response factor of the class, and peak areas of both lipid class and internal standard are included. It is also possible to calculate the concentration of particular lipid species from the relative abundance of their peaks in mass spectra. The advantage of this quantitative method is that it is untargeted and therefore it quantifies all lipid classes without the prerequisite knowledge of certain fragmentation behaviour. In addition, the response factor can be used for later quantitative analysis since is shown to be time-stable.

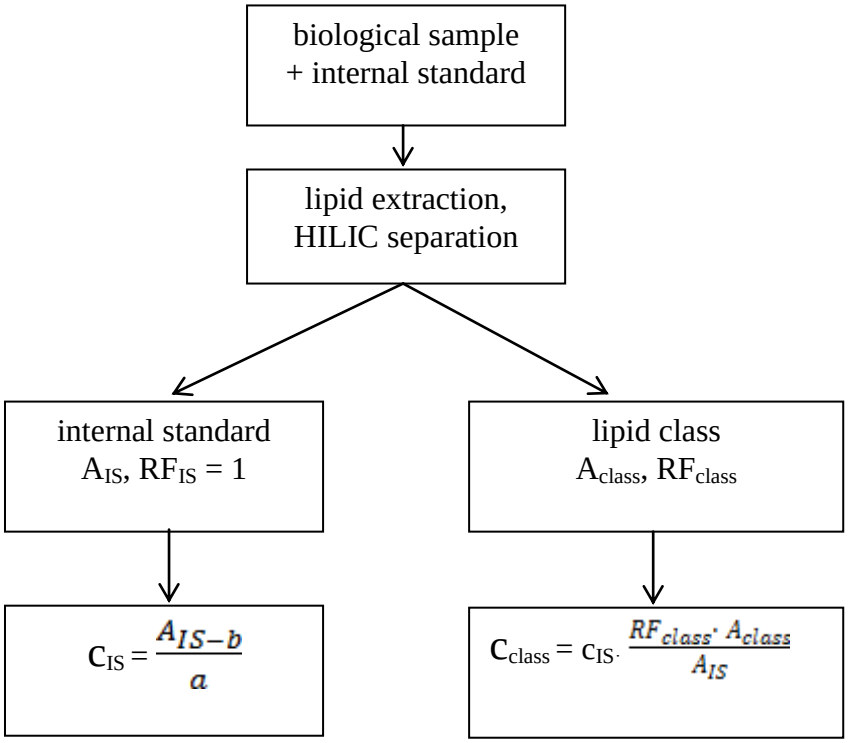


Figure 24: Scheme of lipid class quantification by Cifkova *et al*. (2012), where RF = response factor, IS = internal standard, A = peak area c = concentration, a = slope, b = intercept

# 7 Alzheimer's disease

## 7.1 Introduction

Alzheimer's disease (AD) is a lethal neurodegenerative disease currently affecting 35 million people worldwide (World Health Organization, 2012). Onset is typically after 65 year of age; however, with particular genetic predispositions the disease can occur even earlier (Early-Onset AD, Mendez, 2012). Other than genetic, risk factors can be vascular

(hypertension, obesity, smoking etc.) and psychosocial (mental activity, education, social engagement etc.) (Povova *et al*., 2012).

From the time of diagnosis, it takes about 3-9 years, until the disease reaches its final stage (Querfurth *et* LaFerla, 2010). The early symptoms of the disease confer a variety of behavioural changes (e.g. memory loss, disorientation, confusion). Eventually, self-care becomes impossible and problems with regular physiological functioning result in death (Burns *et* Illife, 2009). Observable neuro-anatomical changes, amyloid plaques, neurofibrillary tangles and loss of neurons are characteristic of AD (Hooijmans *et* Kiliaan, 2008).

As regards treatment, despite the amount of intensive AD research, a cure has still not been found. However, some studies suggest that it may be possible to prevent the disease, or ameliorate symptoms through appropriate diet (e.g. higher intake of polyunsaturated fatty acids present in fish, lower intake of saturated fats, cholesterol) and a healthy life style (physical exercise, no smoking) (Hooijmans *et* Kiliaan, 2008).

## 7.2 Molecular mechanism of AD

In spite of intensive research, the cause of AD is still unclear, however, though a great deal of information on the molecular level is known. Hypotheses concerning the cause of Alzheimer's disease on molecular level are largely based on the overproduction of a specific β-amyloid peptide, and dysfunctional activity of the tau protein.

β-amyloid peptide arises through cleavage of transmembrane amyloid precursor protein (APP) by β- and γ-secretase (composed from presenilin 1 and presenilin 2). Two isoforms of β-amyloid are formed, known as β-amyloid 40 and 42, where β-amyloid 42 is responsible for the creation of extracellular amyloid plaques (Hooijmans *et* Kiliaan, 2008). β-amyloid hypothesis is also supported by observed genetic predispositions to AD, which include mutation in genes for APP, presenilin 1, 2, and the presence of the ε4 allele in gene for apolipoprotein E (Hooijmans *et* Kiliaan, 2008). The effects of β-amyloid protofibril accumulation are unclear, however, some hypotheses suggest, that it could activate microglia - furthering inflammation with the release of cytokines and excitatory amino acids. This in turn would lead to oxidative stress in neurons, with the resulting aggregates potentially blocking the transport in axons and dendrites (Bossy-Wetzel *et al*., 2004).

The second most popular AD hypothesis is based on tau protein. The tau protein normally helps with the assembly and stabilization of microtubules in neural cells, but in AD, it is hyperphosphorylated and functionless. This leads to the disintegration of microtubules and the formation of intracellular aggregates (so called neurofibrillary tangles), which are cytotoxic and cause decrease in axon transport and neuron death (Querfurth *et* LaFerla, 2010).

## 7.3 Lipid biomarkers in AD

One of the main goals of lipidomics is the discovery of biomarkers of particular diseases. Specific lipid molecules that are highly differential between healthy people and those with (or designated as high risk for) the disease are used as biomarkers. The detection of these biomarkers can help to predict the emergence of the disease, or at least aid in early detection. This methodology is applied towards any number of diseases associated with lipids – obesity, atherosclerosis, diabetes, AD and several others (Hu *et al*., 2009).

Many scientists now focus on findings of surrounding AD markers in the lipid group. The affected organ, the brain, is mostly formed by lipids that are also the main components of cytoplasmic membranes (Sagin *et* Sozmen, 2008). One protein especially related to AD is transmembrane APP that is in turn related to other cytoplasmic membrane proteins. This may explain the important role of lipids in the progression of the disease (Grziwa *et al*., 2003). As regards phosphatidylcholines, the lower levels of docosahexaenoic PCs in plasma (Schaefer *et al*., 2006) and elevated PC levels in cerebrospinal fluid (CSF) of AD patients (Walter *et al*., 2004) were observed. Furthermore, decreased ratio of lysophosphatidylcholines/PCs in CSF of AD patients has been observed (Mulder *et al*., 2003).

The cause of these changes is unknown, however it is assumed that the membranes of neurons are degraded during AD and PCs are released from the destroyed membranes to the CSF (Mapstone *et al*., 2014). The connection between CSF and plasma lipid levels remains unclear as the brain lacks the direct contact with the surrounding blood because of the blood-brain barrier. Despite this fact, the blood of the patient is still the most used sample for biomarker detection, since it is easily obtained compared to CSF, which needs to be collected by more invasive methods. However, it is necessary to take into account that the actual lipid levels in the blood can be affected by diet, ethnicity, etc. (Thambisetty *et* Lovestone, 2010).

## 7.4 Chosen lipidomic studies of AD

In 2011, Oresic *et al*. published a metabolomics/lipidomics study of 236 people, divided in 3 groups – healthy, people with mild cognitive impairment (MCI, early state of AD) and AD patients. They observed changes in levels of different metabolites in serum, and a variety of phosphatidylcholines (PCs), ether phospholipids, sphingomyelins and sterols. PCs, which indicated differences between healthy people and AD patients, are shown in the Table I. From other metabolites, changes in 2,4-dihydroxybutanoic acid levels were observed, leading to the hypothesis of hypoxia and oxidative stress during AD.

In 2014, Mapstone *et al*. published a study announcing 10 potential lipid biomarkers of AD detected in human blood. Using untargeted, as well as targeted metabolomics and lipidomics, they observed lowered levels of 10 blood lipids (eight PCs and two acyl carnitines, Table I) in AD patients and people with MCI. From these results, they also predicted, which healthy people will suffer from MCI or AD in next 2-3 years, and which will remain stable, with 90% accuracy. Although the study of Mapstone *et al*. caused a huge wave of interest in the media worldwide (e.g. http://edition.cnn.com/, 2014), this research was only a preliminary cohort study of 525 people older than 70. For confirmation of this AD biomarker hypothesis, it will be necessary to provide an extensive longitudinal study, including people throughout different ethnics and ages.

Earlier in 2014, Whiley *et al*. published plasma lipidomics study of MCI and AD patients using LC-MS and NMR methods. First it was screened by untargeted approach for potential biomarkers in smaller set of plasma of healthy people, people with MCI and AD patients and after that, larger set of 142 plasma samples was analysed by targeted approach for confirmation of suggested biomarkers. Three PCs were lower in AD patients (see Table I), and it is important to note, that changes of two of them were also observed by Mapstone *et al*. (2014) (PC (16:0/22:6) is one of the isobars of PC (38:6) and PC (18:0/22:6) is the isobar of PC (40:6)).

Table I: Potential lipid biomarkers detected by Oresic *et al*., 2011; Mapstone *et al*., 2014 and Whiley *et al*., 2014, where AC – acyl carnitine, PC – phosphatidylcholine, ae – acyl-alkyl chain

| **Potential lipid biomarkers of AD** | | |
|---|---|---|
| **Oresic *et al*., 2011** | **Mapstone *et al*., 2014** | **Whiley *et al*., 2014** |
| **PC (16:0/16:0)** | PC (36:6) | PC (16:0/20:5) |
| **PC (16:0/18:1)** | PC (38:0) | PC (16:0/22:6) |
| **PC (16:0/18:2)** | PC (38:6) | PC (18:0/22:6) |
| **PC (18:0/18:1)** | PC (40:2) | |
| **PC (18:0/18:2)** | PC (40:6) | |
| **PC (16:0/20:3)** | PC ae (40:6) | |
| **PC (O-18:1/16:0)** | PC (40:1) | |
| **PC (O-18:1/18:2)** | Lyso PC (18:2) | |
| | Propionyl AC (C3) | |
| | C16:1-OH | |

*EXPERIMENTAL PART*

# 1 INTRODUCTION

Lipidomics of Alzheimer‘s disease is intensively studied field nowadays. Since the first step of reliable lipid characterization and quantification is the development of suitable analytical method, the main part of this thesis will focus on that. In addition, the practical part includes the quantification of chosen lipids in plasma, calculations of extraction recoveries using different extraction solvents, matrix effect of plasma and limits of detection and quantification for chosen lipids.

The lipids for the analysis were chosen from the group of phosphatidylcholines (PCs), since they showed changed levels during metabolomics study of AD by Oresic *et al*. (2011). Eight PCs (see Table I in Chapter 7.4) with different levels in AD patients were observed, and from them, four were chosen for our analysis since their standards were available commercially - PC (16:0/16:0), PC (18:0/18:1), PC (18:1/18:2) and PC (16:0/18:2). In addition, PC (17:0/17:0) was chosen as the internal standard (IS), since it is not naturally present in human plasma. Chosen PCs were analysed by five mass spectrometric techniques while the final analyses were performed using quadrupole ion trap (Q-Trap) mass spectrometer.

# 2 MATERIAL AND METHODS

## 2.1 Biological material

- Human plasma and pooled plasma from 6 individuals

## 2.2 Instruments

- HPLC:
  - 1200 Infinity Series (Agilent Technologies, USA)
  - 1290 Inifinity Series (Agilent Technologies, USA)
  - Dionex Ultimate 3000 RSLC (Thermo Scientific, USA)
- Columns:
  - Cortecs C18, 2.7 μm, 2.1 x 100 mm, 90Å (Waters, Ireland)
  - Zorbax Eclipse XDB C18, 1.8 μm, 2.1 x 100 mm (Agilent Technologies, USA)
  - X Bridge C18, 2.5 μm, 2.1 x 50 mm (Waters, Ireland)
  - Kinetex C18, 2.6 μm, 2.1 x 50 mm (Phenomenex, USA)
- MS:
  - Triple quadrupole mass spectrometer - 6410 Triple Quadrupole LC/MS; 6495 Triple Quadrupole LC/MS (Agilent Technologies, USA)
  - Linear ion trap mass spectrometer - LTQ Finnigan (Thermo Scientific, USA)
  - Quadrupole time-of-flight mass spectrometer - 6540 UHD Accurate – Mass QTOF (Agilent Technologies, USA)
  - Quadrupole ion trap – QTRAP 5500 (AB Sciex, USA)
- Vortices:
  - Vortex Genie 2 (Scientific Industries, USA)
  - V1 plus (BioSan, Lithuania)
- Balances:
  - PB602-S (Mettler Toledo, USA)
  - AB-S (Mettler Toledo, USA)
  - BP 221 S (Sartorius, Germany)
- Pipettes:
  - VoluMate Liquisystems (Mettler Toledo, USA)
  - Finnpipette (Thermo Scientific, USA)
  - Eppendorf Research Plus (Eppendorf, Germany)
- Centrifuges:
  - 5804 R (Eppendorf, Germany)
  - Mikro 120 (Hettich, Germany)

- Syringe: - 2 ml (Terumo, Japan)
- Needle: - 1.2 x 40 mm (Terumo, Japan)
- Syringe filter: - Acrodisc CR13 PTFE, 0.2 μm (Pall, USA)
- Ultrasonic cleaner: - 3510 (Branson, USA)
- PS-40 (Jeken, China)

## 2.3 Standards and chemicals

- PC (16:0/16:0) - 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine - $C_{40}H_{80}NO_8P$ (Avanti Polar Lipids, USA)
- PC (18:0/18:1) - 1-octadecanoyl-2-(11Z-octadecenoyl)-*sn*-glycero-3-phosphocholine - $C_{44}H_{86}NO_8P$ (Avanti Polar Lipids, USA)
- PC (17:0/17:0) - 1,2-diheptadecanoyl-s*n*-glycero-3-phosphocholine - $C_{42}H_{84}NO_8P$ (Avanti Polar Lipids, USA)
- PC (16:0/18:2) - 1-hexadecanoyl-2-(10E,12Z-octadecadienoyl)-*sn*-glycero-3-phosphocholine - $C_{42}H_{80}NO_8P$ (Avanti Polar Lipids, USA)
- PC (18:0/18:2) - 1-octadecanoyl-2-(10Z,12Z-octadecadienoyl)-*sn*-glycero-3-phosphocholine - $C_{44}H_{84}NO_8P$ (Avanti Polar Lipids, USA)
- L-α-phosphatidylcholine – Egg (Avanti Polar Lipids, USA)
- Methanol - $CH_3OH$ (Fisher Scientific, UK; Sigma Aldrich, USA)
- Ethanol - $CH_3CH_2OH$ (Altia Oyj, Finland; Merck KgaA, Germany)
- 98-100% formic acid - $CH_3COOH$ (Sigma Aldrich, USA)
- Ammonium acetate - $CH_3COONH_4$ (Riedel-de Haën, USA)
- Acetonitrile - $CH_3CN$ (VWR International, USA)
- Isopropyl alcohol - $(CH_3)_2CHOH$ (Sigma Aldrich, USA)
- Ammonium fluoride - $NH_4F$ (Sigma Aldrich, USA)
- Dimethylsulfoxid – $(CH_3)_2SO$ (Sigma Aldrich, USA)
- LC-MS water

## 2.4 Work procedure

### 2.4.1 Preparation of PC standard stock solutions

A certain amount of particular PC standard powder was weighed and dissolved in ethanol (EtOH) for obtaining 5 mM stock solution. All prepared PC standard stock solutions were stored in glass bottles in -20 °C prior the analysis. Working solutions of PCs were prepared by dilution (or mixing and dilution) of standard stock solutions in methanol (MeOH) to chosen concentrations.

### 2.4.2 HPLC analysis and settings

Separation of the chosen PCs was obtained using reversed phase high performance liquid chromatography with mass spectrometric detection. For all MS instrumentation except quadrupole time-of-flight (QTOF), the HPLC separation conditions including selection of column, its temperature, mobile phases, the gradient, flow rate, and injection volume of sample were optimized. For QTOF analysis, HPLC conditions typical for metabolomics analysis were used.

- column selection

Four different chromatographic C18 columns were tested for PC separation – the columns being Cortecs C18, Kinetex C18, X Bridge C18 and Zorbax Eclipse XDB C18. For the final analyses on Q-Trap, a 10 cm long Cortecs C18 column with the temperature of 50 °C was used.

- mobile phase selection

Several mobile phases including aqueous (A) and organic (B) solutions were tested, see Table II. For the final Q-Trap analysis, 0.1% formic acid in water with 5% of MeOH as mobile phase A, and MeOH with 0.1% formic acid and 5% LC-MS water as mobile phase B, were chosen.

Table II: Tested mobile phases

| **mobile phase A (aqueous)** | **mobile phase B (organic)** |
|---|---|
| *0.1% formic acid in water + 5% of methanol* | *methanol + 0.1% formic acid + 5% LC-MS water* |
| 0.1% formic acid in water | methanol + 0.1% formic acid |
| 5 mM ammonium acetate in water | acetonitrile |
| 10 mM ammonium acetate in water | acetonitrile: isopropanol (5:2) |
| 10 mM ammonium acetate in water + 0.1% formic acid | 0.2 mM ammonium fluoride |

- gradient selection

Separation of PCs was obtained using gradient elution. Different elution programs were tested during the method development. The gradient chosen for the final analysis with Q-Trap is shown in the Figure 25. The gradient starts on 60% of mobile phase B and achieves 100% in 1.5 min. After that, isocratic elution for 9 min occurs and the gradient is changed back to 60% of B in 0.1 min, and is left there for 3.4 min. The total time of analysis was 14 minutes. The flow rate of mobile phase in final Q-Trap analysis was 0.5 ml/min, with injection volume of 1 µl.

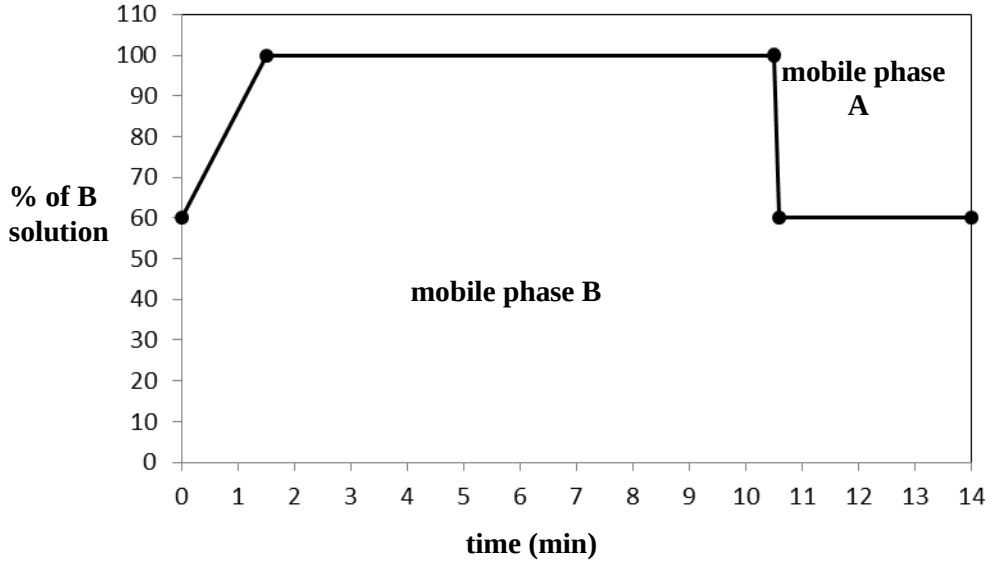


Figure 25: Final chromatographic gradient

### 2.4.3 MS analysis and settings

Detection of PCs was obtained by five MS instruments - quadrupole time-of-flight (QTOF), linear ion trap (LTQ), quadrupole ion trap (Q-Trap) and by two triple quadrupoles (Triple Quad 6410, Triple Quad 6495). For every instrument except QTOF, the detection conditions for PCs were optimized (analysis on QTOF was performed using metabolomics settings).

The final measurements were done by Q-Trap mass spectrometer. The *m/z* of precursor and product ions of PCs in positive and negative multiple reaction monitoring modes (ESI+ MRM, ESI- MRM) and the collision energies suitable for PC fragmentation are shown in the Table III. The curtain gas flow was 30 instrument units, temperature of ion source 350 °C and ion spray voltage -4500 V.

Data acquired from QTOF, Triple Quad 6410 and Triple Quad 6495 analyses was processed using MassHunter software, data from LTQ analyses by Xcalibur software and data from Q-Trap analyses by Analyst software.

Table III: The *m/z* of precursor and product ions of PCs and collision energies for ESI+ and ESI- MRM acquired using Q-Trap

| **PC** | **precursor ion in ESI+ MRM** | **product ion in ESI+ MRM** | **collision energy (V)** | **precursor ion in ESI- MRM** | **product ion in ESI- MRM** | **collision energy (V)** |
|---|---|---|---|---|---|---|
| PC (16:0/16:0) | 734.5 | 184.1 | 39 | 778.5 | 255.1 | 50 |
| | | | | | 480.3 | 46 |
| PC (18:0/18:2) | 786.3 | | 41 | 830.5 | 283.3 | 52 |
| | | | | | 279.3 | 52 |
| PC (16:0/18:2) | 758.3 | | 39 | 802.5 | 255.2 | 50 |
| | | | | | 279.2 | 50 |
| PC (18:0/18:1) | 788.4 | | 41 | 832.5 | 281.3 | 50 |
| | | | | | 283.2 | 56 |
| PC (17:0/17:0) | 762.3 | | 41 | 806.5 | 269.1 | 52 |

### 2.4.4 Extraction of PCs from human plasma

Two phosphatidylcholine extraction methods were tested, firstly by pure acetonitrile (samples for Triple Quad 6495, LTQ and QTOF) and secondly by 50% methanol-ethanol (final samples for Q-Trap).

First, the extraction by acetonitrile (ACN) was tested, since it represents classic protein-precipitative method used in metabolomics analysis. 90 µl of plasma was mixed with 360 µl of ice cold ACN while adding it in drops during sample vortexing. The solution was centrifuged for 10 minutes with 14 000 rpm for protein precipitant settlement. The supernatant was taken by the needle into syringe and squeezed through filter to remove unwanted particles.

The other extraction was made by mixing of 50 µl of plasma with 950 µl of 50% MeOH-EtOH. The solution was mixed, sonicated for 5 minutes and left in the freezer for 15 minutes. Then, the mixture was centrifuged for 5 min with 14 000 rpm and the supernatant was collected.

### 2.4.5 Extraction recovery, matrix effect test of plasma

The extraction recovery was tested using internal standard (IS) PC (17:0/17:0), since this PC is not naturally present in human plasma. 45 µl of pooled plasma and 5 µl of IS solution (200 µM) or 5 µl of pure MeOH was mixed. To the solution, 950 µl of 50% MeOH-EtOH (or ACN) was added and mixed. After sonication, freezing and centrifugation, 5 µl of IS solution or 5 µl of pure MeOH was added, meaning, to the samples already containing IS, only MeOH was added and to the samples with MeOH, IS was added.

Six samples with IS added before the extraction and six samples with IS added after extraction with 50% MeOH-EtOH solvent were prepared, and for comparison, the same number of samples using ACN instead of 50% MeOH-EtOH was obtained. For the matrix effect test, six samples using water instead of plasma were prepared while three of them were „extracted“ by 50% MeOH-EtOH and other three by ACN.

### 2.4.6 Quantification of PCs by standard addition method

The chosen PCs in plasma were quantified using standard addition method. First, the suitable concentrations of PC standards for spiking of plasma were obtained by creation of the calibration curve of the four PC standards in MeOH, with concentrations 0, 50, 100, 250, 500, 1000 and 2000 nM.

Then, the samples for the quantification of the four PCs in chosen human plasma were prepared as follows - first blank plasma was extracted using 50% MeOH-EtOH for obtaining 5000x diluted sample. Then 50 µl of this sample was spiked by 10 µl of 0.5, 1, 2, 5, 10 and 20 µM mix of the four PC standards, and 40 µl of IS (250 nM) was added. The final dilution of plasma was therefore 10 000 times and the concentrations of the four PC standards 0, 50, 100, 250, 500, 1000 and 2000 nM. From detected peak areas of particular PCs in spiked plasma samples and the peak areas of IS, the calibration curve was created and the quantification of the four PCs in un-spiked human plasma was obtained.

# 3 RESULTS

Phosphatidylcholines in the form of commercial standards as well as naturally present compounds in Egg phosphatidylcholine mix and plasma samples were separated by RP-LC and detected by five MS instruments including Q-Trap, QTOF, LTQ and Triple Quads 6410 and 6495. The final analyses were acquired using Q-Trap. All PCs were observed as hydrogen adducts in positive ionization mode (ESI+) and as formic acid adducts in negative ionization mode (ESI-). The list of analysed PCs with their exact masses and adducts in ESI+ and ESI- is shown in the Table IV.

Table IV: The exact masses of PCs and their adducts in ESI+, ESI- (calculated by MassHunter software)

| **Phosphatidylcholine** | **Exact mass** | **[M+H]+** | **[M+COOH]$^-$** |
|---|---|---|---|
| PC (16:0/16:0) | 733.5622 | 734.5694 | 778.5604 |
| PC (17:0/17:0) | 761.5935 | 762.6007 | 806.5917 |
| PC (16:0/18:2) | 757.5622 | 758.5694 | 802.5604 |
| PC (18:0/18:1) | 787.6091 | 788.6164 | 832.6073 |
| PC (18:0/18:2) | 785.5935 | 786.6007 | 830.5917 |

### 3.1.1 Triple Quad 6410 and Triple Quad 6495 analysis of PCs

- Triple Quad 6410 analysis

With Triple Quad 6410, the manual optimization of the parameters was done first. It included searching for the best fragmentor voltages and collision energies for fragmentation of PC standards. One of the tests for suitable fragmentation voltage in ESI- is shown in the Figure 26, where the voltages in range 120-200 V for PC (16:0/16:0) were tested. The highest ion intensity of PC (16:0/16:0) was obtained with fragmentor voltage between 160-180 V. For all PCs, the average value was determined - for measurements in ESI-, the fragmentor voltage was 170 V and for ESI+ 240 V.

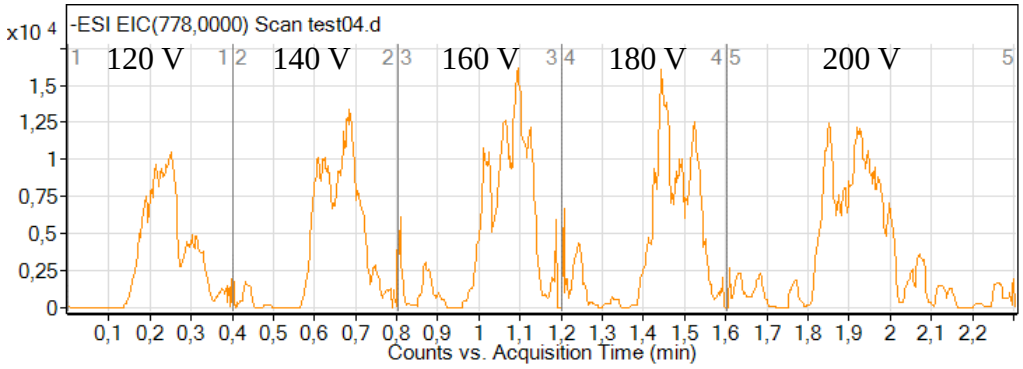


Figure 26: Fragmentor voltage test for PC (16:0/16:0) in ESI-

In the Figure 27 there is an example of the test for suitable collision energy in ESI+, where the energies in range 28-44 V for PC (16:0/16:0) were tested. The most intensive peaks of 184 Da fragments of PC (16:0/16:0) are coming with collision energy about 36 V. The average value of collision energy for all PCs was determined as 32 V, in both ESI+ and ESI-.

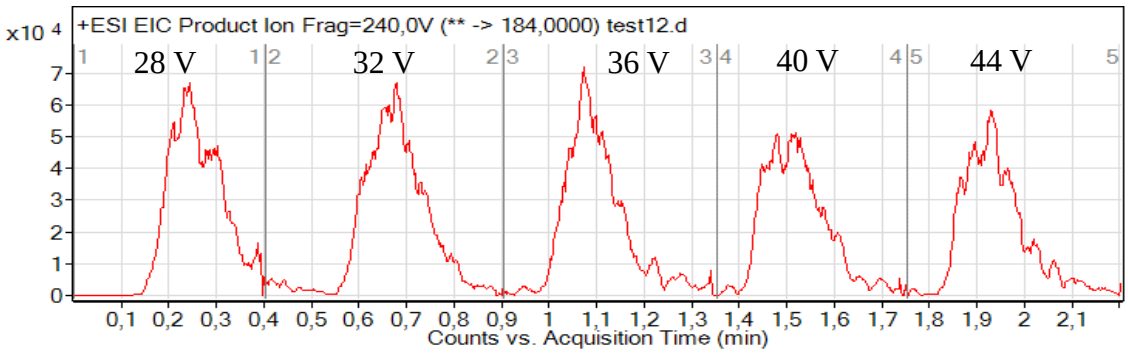


Figure 27: Collision energy test for PC (16:0/16:0) in ESI-

After instrument optimization, the samples including the mix of the four PC standards and IS in MeOH and commercial Egg phosphatidylcholine mix, were measured. Both samples were analysed in both ESI+ and ESI- MRM modes.

In the Figure 28 are ESI+ MRM chromatograms of 100 nM mix of the four PC standards and IS in MeOH. In ESI+, all PCs are characterized by the fragment of *m/z* 184 Da belonging to the phosphocholine head.

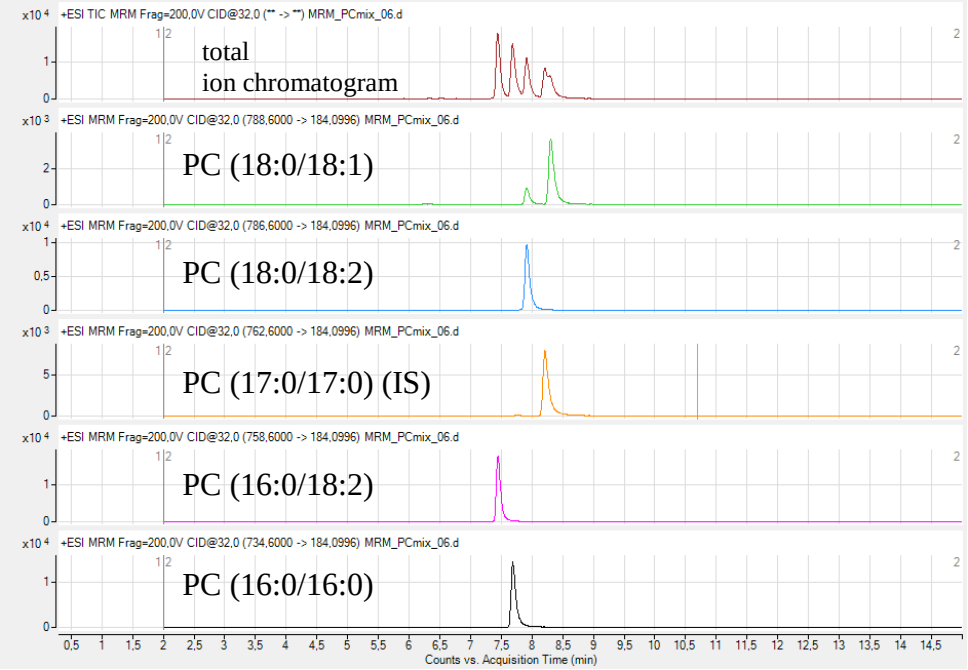


Figure 28: ESI+ MRM chromatograms of the four 100 nM PC standards + IS in MeOH

In the Figure 29, ESI- MRM chromatograms of chosen PCs in commercial Egg phosphatidylcholine mix are shown. In general, when using ESI-, each PC is characterized by two fragments belonging to the fatty acids, which are detected as $[M-H]^-$ ions (where M represents the fatty acid molecule). Basic fatty acids with their exact masses and *m/z* of $[M-H]^-$ ions are shown in the Table V.

Therefore, several ESI- MRM transitions were set for the detection of PCs in Egg PC mix, e.g. for determination of PC (16:0/18:2), one transition representing palmitic acid C16:0 (*m/z* 802.6 → 255.0) and the second for linoleic acid C18:2 (*m/z* 802.6 → 279.0) were provided. The Figure 29 shows that in Egg PC mix the detected PCs were PC (16:0/16:0), (16:0/18:2), (16:0/18:1), (18:0/18:1) and (18:0/18:2), and compared to that, PC (17:0/17:0) was not detected. This result corresponds to PCs observed in egg phosphatidylcholine fraction by Smith *et* Jungalwala (1981).

Table V: Exact masses of fatty acids and their adducts in ESI-

(http://www.lipidmaps.org/)

| Fatty acid | Abbreviation | Exact mass | $[M-H]^-$ |
|---|---|---|---|
| palmitic acid | C16:0 | 256.2402 | 255.2330 |
| margaric acid | C17:0 | 270.2559 | 269.2486 |
| stearic acid | C18:0 | 284.2715 | 283.2643 |
| oleic acid | C18:1 | 282.2559 | 281.2486 |
| linoleic acid | C18:2 | 280.2402 | 279.2330 |

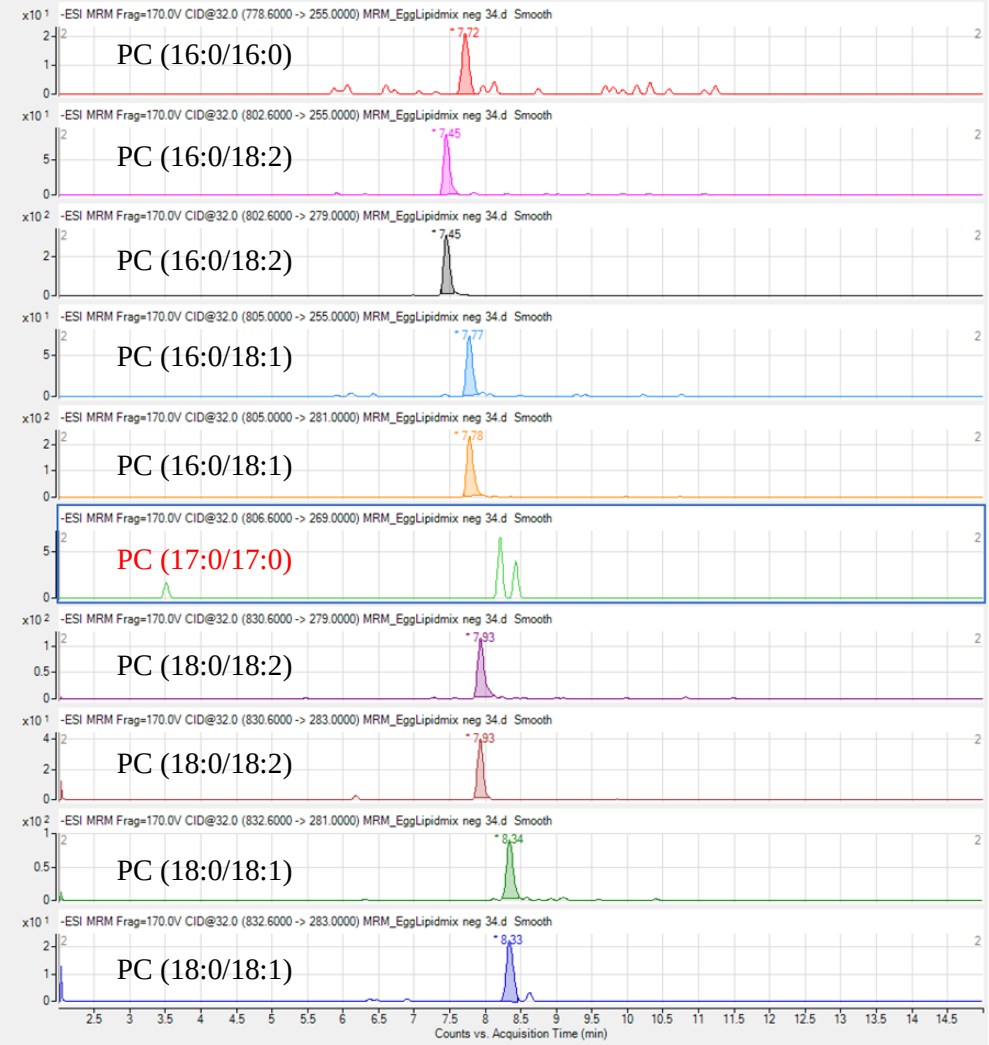


Figure 29: ESI- MRM chromatograms of chosen PCs in Egg phosphatidylcholine mix

- Triple Quad 6495 analysis

PCs were measured also on Triple Quad 6495 with the aim to compare two Triple Quads. Using Triple Quad 6495, the optimization of fragmentor voltages and collision energies for particular PC was performed automatically and the samples were measured only in ESI- MRM mode.

The analysed samples included mix of the four PC standards and IS in MeOH and plasma samples extracted by ACN. In the Figure 30 are ESI- MRM chromatograms of the four PCs detected in human plasma with 2 μM IS.

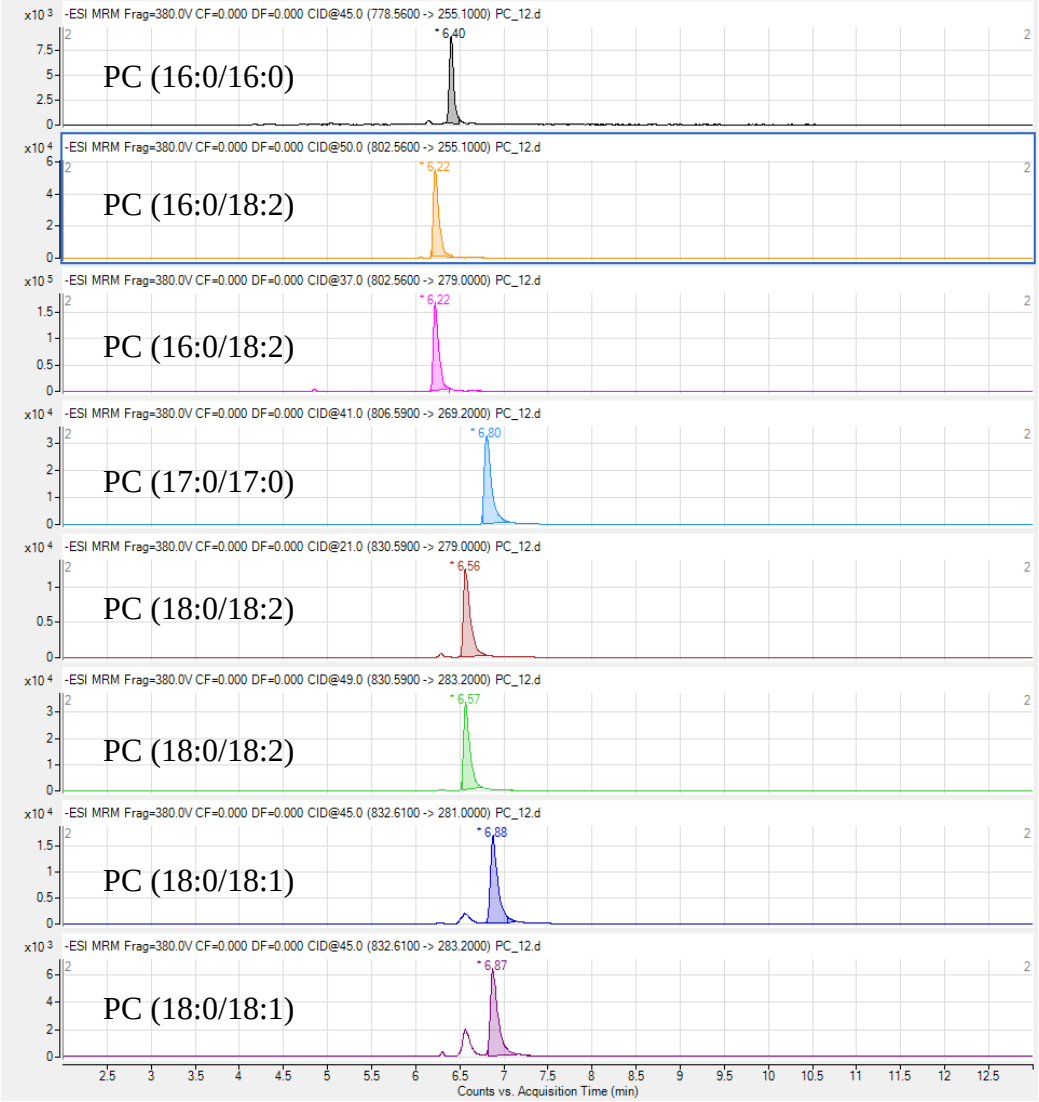


Figure 30: ESI- MRM chromatograms of PCs in plasma + 2 μM IS

### 3.1.2 QTOF analysis of PCs

The QTOF analysis of PCs was performed with the aim to detect the four PC standards in human plasma if using metabolomics approach of the Metabolomics Centre of the University of Eastern Finland in Kuopio. The measured samples included mix of the four PC standards with IS and by ACN extracted plasma.

In the Figure 31 there are ESI- full scan chromatograms of the four PCs detected in plasma with 1 µM IS. The metabolomics approach (including Zorbax Eclipse XDB C18 column) was not very suitable for PC detection, since it did not provide good peak shapes and separation of PCs, however, using MS/MS analysis, it was possible to observe fragmentation behaviour of PCs.

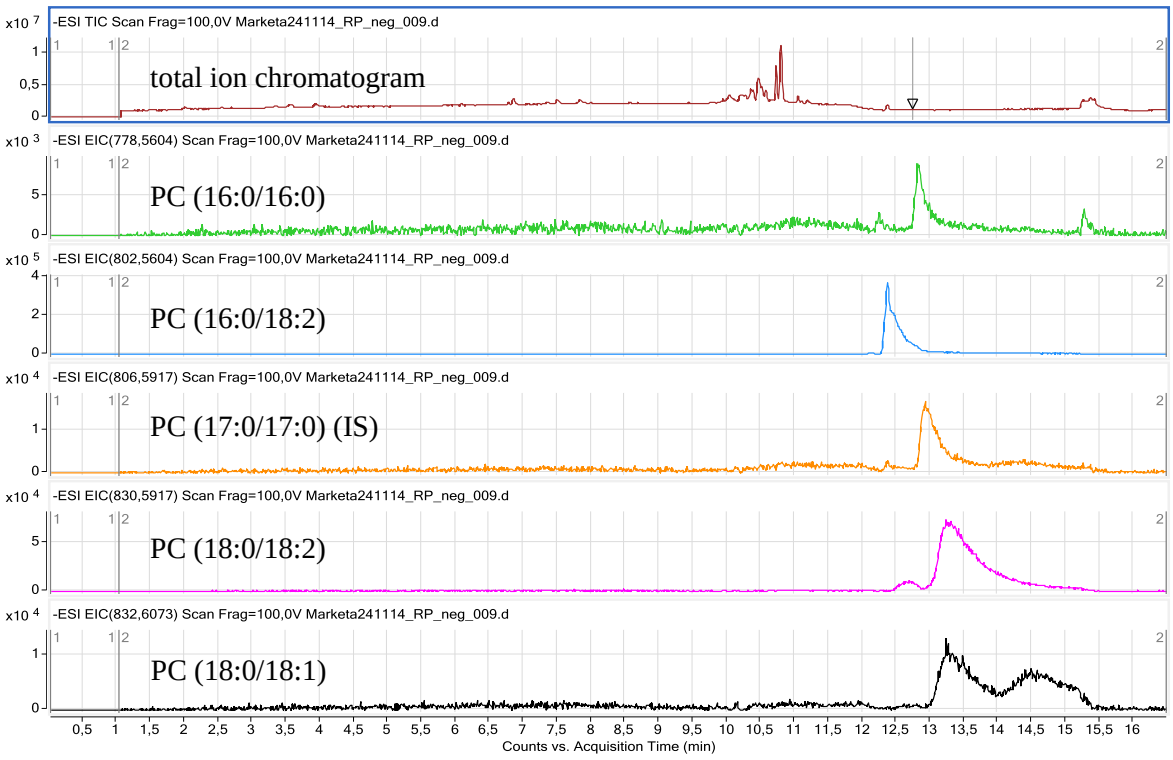


Figure 31: ESI- full scan chromatograms of PCs in plasma + 1 µM IS

In the Figure 32 is shown the ESI- MS/MS spectrum of PC (16:0/18:2). Chosen PC was detected as formic acid adduct and the first fragment was assigned as demethylated PC obtained by the cleavage of acetic acid from the $[M+COOH]^-$ adduct. Two other fragments were identified as fatty acyls – palmitic (C16:0) and linoleic acid (C18:2).

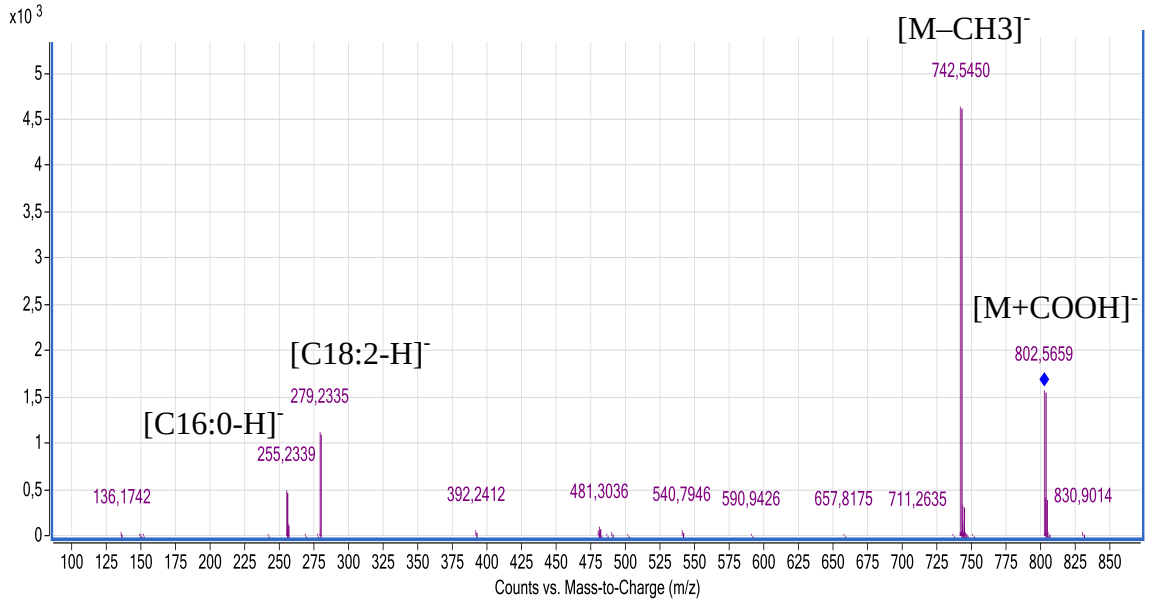


Figure 32: ESI- MS/MS spectrum of PC (16:0/18:2)

### 3.1.3 LTQ analysis of PCs

For further study of fragmentation behaviour, PCs were analysed using LTQ ion trap in negative ionization mode. After the optimization of collision energies for $MS^2$ and $MS^3$ analyses of the PCs, the mix of the four PC standards and IS in MeOH, and plasma samples extracted by ACN were measured.

In the Figure 33 there are $MS^3$ chromatograms of the four PC standards with IS in MeOH. PCs were also detected as formic acid adducts in full scan MS and after the fragmentation, the prevailing fragments in $MS^2$ were demethylated PCs, and in $MS^3$ the fatty acyls, resolving particular PCs between each other.

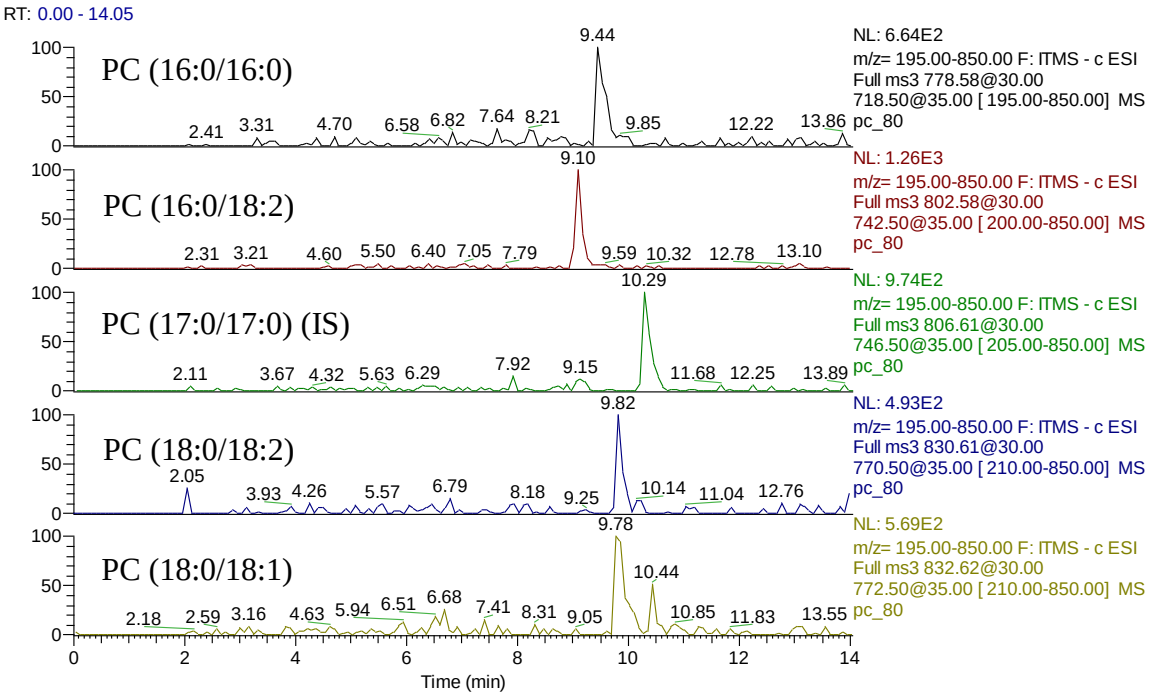


Figure 33: ESI- $MS^3$ chromatograms of the four 500 nM PC standards + 1µM IS in MeOH

### 3.1.4 Q-Trap analysis of PCs

The final analyses of the four chosen PCs were performed using Q-Trap mass spectrometer. All samples (the four PC standards with IS in MeOH, plasma samples) were measured in ESI- MRM mode.

In the Figure 34 is shown the ESI- MRM chromatogram of the four 100 nM PC standards with IS. Although all PCs had the same concentration in the mixture, different ion intensities can be observed, since the PCs with shorter fatty acyl chains and more double bonds provide higher ionization efficiency (Koivusalo *et al*, 2001). This fact can be shown on the example of PC (18:0/18:2) and PC (18:0/18:1), where, related to the number of double bonds, PC (18:0/18:2) shows higher intensity than PC (18:0/18:1). As regards the length of the chain, a good example would be the difference between PCs (16:0/16:0) and (17:0/17:0), where the peak of PC (16:0/16:0) is more intensive than the peak of PC (17:0/17:0).

It was also possible to observe the elution order of the PCs based on their hydrophobic carbon number (HCN). HCN is calculated from the number of carbons and double bonds (one double bond lowers the HCN by 1.8 units and the other by 1.6, 1.4; 1.2 and 1 unit). The lower the HCN, the smaller is the retention time (Smith *et* Jungalwala, 1981). After recalculation of HCN of PCs and sorting them from the smallest to the largest, the elution order is: PC (16:0/18:2) – 30.8, PC (16:0/16:0) – 32.0, PC (18:0/18:2) – 32.8, PC (17:/17:0) – 34.0, PC (18:0/18:1) – 34.2 (see Figure 34).

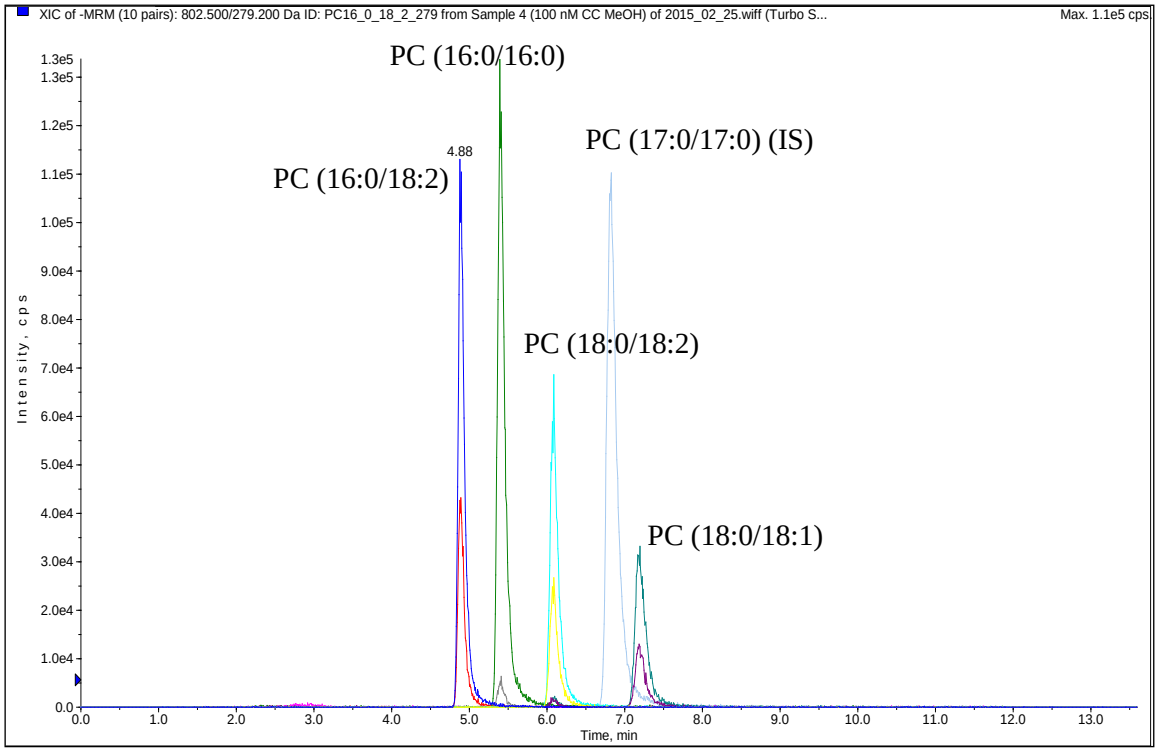


Figure 34: ESI- MRM chromatogram of 100 nM 4 PC standards + IS in MeOH

### 3.1.5 Extraction recovery test and matrix effect test

Extraction recovery test was performed by spiking of pooled plasma by PC 17:0/17:0 (IS) before and after extraction. Two extraction solvents were tested, 50% MeOH-EtOH and ACN, and the test for matrix effect of plasma was performed by replacing the plasma by water. The calculations of extraction recoveries and matrix effect were done according to Matuszewski *et al*. (1998).

In Tables VI, VII and VIII are detected peak areas for PC (17:0/17:0) in plasma and water samples, with calculated average peak areas, coefficients of variation (CV) and average standard deviations (SD).

Table VI: Peak areas of PC (17:0/17:0) (IS) in 50% MeOH-EtOH extracted samples

| **plasma sample number** | IS added: | |
|---|---|---|
| | before extraction | after extraction |
| | **peak area of IS** | |
| 1 | 4.10E+05 | 5.42E+05 |
| 2 | 5.14E+05 | 4.74E+05 |
| 3 | 4.71E+05 | 4.80E+05 |
| 4 | 4.90E+05 | 5.10E+05 |
| 5 | 4.95E+05 | 4.95E+05 |
| 6 | 4.85E+05 | 5.09E+05 |
| average peak area | **4.78E+05** | **5.02E+05** |
| CV | 3.59E+04 | 2.46E+04 |
| average SD | **6.21 %** | |

Table VII: Peak areas of PC (17:0/17:0) (IS) in ACN extracted samples

| **plasma sample number** | IS added: | |
|---|---|---|
| | before extraction | after extraction |
| | **peak area of IS** | |
| 1 | 1.31E+05 | 4.99E+05 |
| 2 | 1.44E+05 | 5.20E+05 |
| 3 | 1.85E+05 | 6.37E+05 |
| 4 | 1.55E+05 | 5.92E+05 |
| 5 | 1.56E+05 | 6.17E+05 |
| 6 | 1.71E+05 | 5.58E+05 |
| average peak area | **1.57E+05** | **5.71E+05** |
| CV | 1.91E+04 | 5.45E+04 |
| average SD | **10.87 %** | |

Table VIII: Peak areas of PC (17:0/17:0) (IS) in water extracted by 50% MeOH-EtOH and ACN

| water sample number | extracted by | peak area of IS |
|---|---|---|
| 1 | 50% MeOH-EtOH | 5.72E+05 |
| 2 | | 5.96E+05 |
| 3 | | 5.28E+05 |
| average peak area | | **5.65E+05** |
| CV | | 3.45E+04 |
| SD | | **6.10 %** |
| 1 | ACN | 4.81E+05 |
| 2 | | 5.72E+05 |
| 3 | | 5.59E+05 |
| average peak area | | **5.37E+05** |
| CV | | 4.92E+04 |
| SD | | **9.16 %** |

- Calculation of extraction recovery:

$$\text{extraction recovery (\%)} = \frac{\text{average peak area of IS added before extraction}}{\text{average peak area of IS added after extraction}} \cdot 100$$

Extraction recovery for 50% MeOH-EtOH extraction: (4.78E+05/5.02E+05) · 100 = 95.18 %
Extraction recovery for ACN extraction: (1.57E+05/5.71E+05) · 100 = 27.52 %

Including the average SD, the extraction recovery for 50% MeOH-EtOH extraction was **95.2 % ± 6.2 %** and the extraction recovery for ACN extraction was **27.5 % ± 10.9 %**.

- Calculation of the matrix effect of plasma:

$$\text{matrix effect (\%)} = \frac{\text{average peak area of IS added after extraction}}{\text{average peak area of IS added to solvent}} \cdot 100$$

Matrix effect of plasma for 50% MeOH-EtOH extraction (if using water as a solvent): (5.02E+05/5.65E+05) · 100 = 88.74 %
Matrix effect of plasma for ACN extraction: (5.71E+05/5.37E+05) · 100 = 106.17 %

Including the average SD (calculated from average SD of peak areas of IS in water and plasma spiked after extraction), the matrix effect of plasma calculated from 50% MeOH-EtOH extraction was **88.7 % ± 5.5 %**, while from ACN extraction **106.2 % ± 9.4 %**.

### 3.1.6 Quantification of PCs in human plasma

The four PCs (PC (16:0/16:0), PC (16:0/18:2), PC (18:0/18:1), PC (18:0/18:2)) were quantified in human plasma by standard addition method. The standard addition method was performed by spiking of 10 000 times diluted plasma by 6 concentrations of the four PC standards and by IS with constant concentration.

For determination of spiking concentrations, the calibration curve of the four PC standards in MeOH was first acquired (chosen calibration curve of PC (16:0/18:2) is shown in the Figure 35). After verifying the linearity of instrument response for chosen concentrations, plasma sample was spiked by a mix of the four PC standards (to final concentrations 0, 50, 100, 250, 500, 1000, 2000 nM) and for each quantified PC, two ESI-MRM transitions were set. The example of calibration curve of PC (16:0/18:2) for product ion 279.2 in ESI- MRM in spiked plasma is shown in the Figure 36.

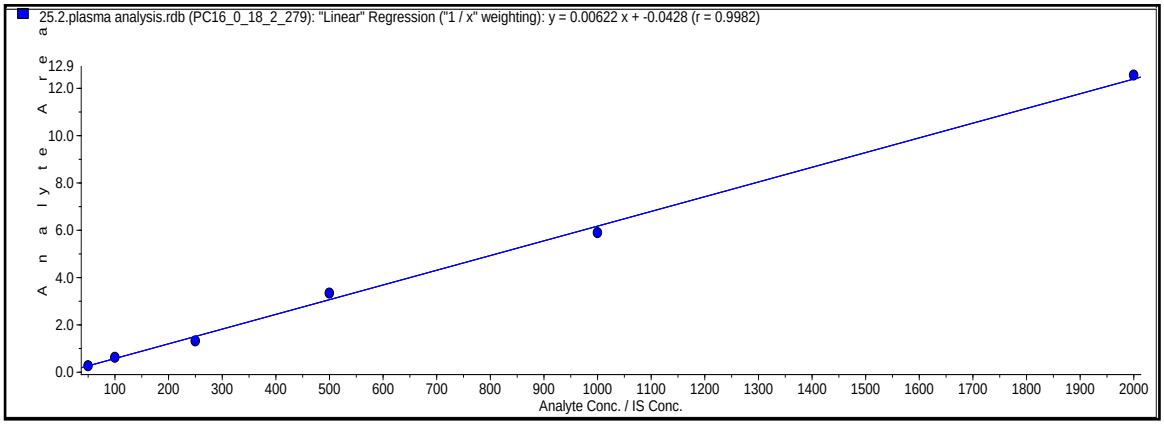


Figure 35: Calibration curve of PC (16:0/18:2) in MeOH

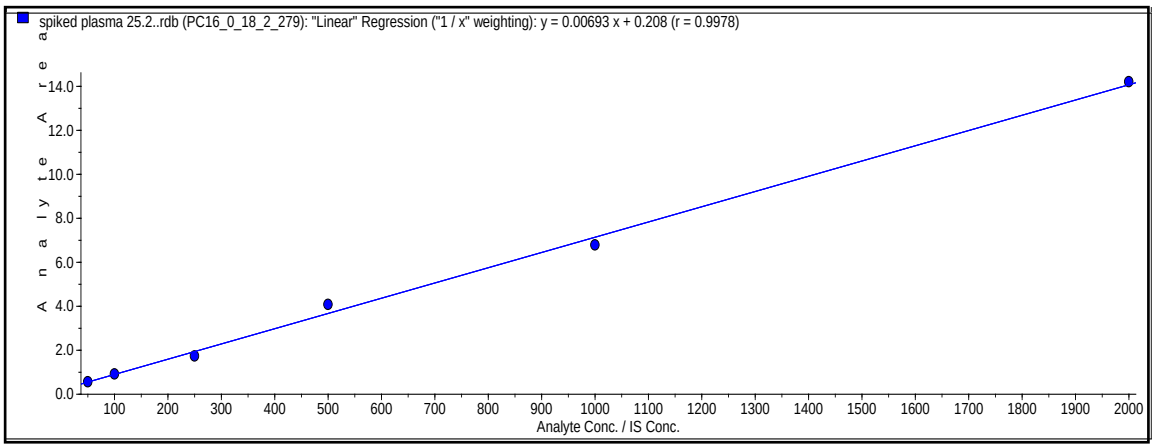


Figure 36: Calibration curve of PC (16:0/18:2) in spiked plasma

- Calculation of the PC concentrations in plasma

The concentration of a chosen PC in human plasma was calculated from linear regression line, acquired from calibration curve of chosen PC in plasma (spiked by known concentrations of the four PC standards).

For example, for PC (16:0/18:2) and ESI- MRM transition *m/z* 802.5 → 279.2, the linear regression line was y = 0.00693x + 0.208. Then "y" was substituted by 0 (extrapolation of calibration curve to zero response) and the unknown concentration of PC in un-spiked plasma was calculated as "x" (30.01 nM for PC (16:0/18:2)). Since the plasma was 10 000 times diluted prior the analysis, the initial concentration of PC 16:0/18:2 in plasma was 10 000 times higher – 300.1 μM. The linear regression lines and calculated concentrations of all four PCs are shown in the Table IX.

Table IX: Quantification of PCs in plasma

| PC | ESI- MRM product ion | linear regression line y = | plasma concentration (µM) |
|---|---|---|---|
| PC (16:0/16:0) | 255.1 | 0.00939x – 0.0388 | 41.3 |
| | 480.3 | 0.00039x – 0.00234 | 60.6 |
| PC (18:0/18:1) | 281.3 | 0.00349x – 0.0024 | 6.9 |
| | 283.2 | 0.00132x + 0.00178 | 13.5 |
| PC (18:0/18:2) | 279.3 | 0.00507x + 0.0873 | 172.2 |
| | 283.3 | 0.00209x + 0.0032 | 15.3 |
| PC (16:0/18:2) | 279.2 | 0.00693x + 0.208 | 300.1 |
| | 255.2 | 0.00283x + 0.0619 | 218.7 |

### 3.1.7 Limit of detection and Limit of quantification of PCs

For comparison of two Triple Quads and Q-Trap instrument, limits of detection (LOD) and quantification (LOQ) were calculated. LOD is characterized as the lowest signal which can be resolved from the noise, while LOQ is the lowest signal which can be measured with required precision of the method (Snyder *et al*., 2010).

LOD and LOQ for all instruments were calculated for PC (16:0/16:0) in MeOH, measured in ESI- MRM mode. From the intensity of noise and chosen peak, the signal to noise ratio (S/N) was automatically calculated. Using S/N and the fact, that LOD should be three times higher, and LOQ ten times higher than noise level (Snyder *et al*., 2010), the LOD and LOQ were calculated related to known PC concentration (Table X). As regards Q-Trap, the LOD and LOQ were calculated for all four PCs in MeOH and plasma to known concentrations calculated by previous quantification (Table XI, XII).

Table X: LOD and LOQ for PC 16:0/16:0 in MeOH

| PC | product ion in ESI-MRM | concentration (nM) | instrument | S/N | LOD (nM) | LOQ (nM) |
|---|---|---|---|---|---|---|
| PC (16:0/16:0) | 255.1 | 100 | Triple Quad 6410 | 244.4 | 1.23 | 4.09 |
| | | | Triple Quad 6495 | 50.9 | 5.89 | 19.65 |
| | | | Q-Trap | 308.4 | 0.97 | 3.24 |

Table XI: LOD and LOQ for PC standards in MeOH (Q-Trap analysis)

| PC | product ion in ESI- MRM | concentration (nM) | S/N | LOD (nM) | LOQ (nM) |
|---|---|---|---|---|---|
| PC (16:0/16:0) | 255.1 | 100 | 308.4 | 0.97 | 3.24 |
| | 480.3 | | 47.4 | 6.33 | 21.09 |
| PC (18:0/18:1) | 281.3 | | 99.2 | 3.02 | 10.08 |
| | 283.2 | | 64.5 | 4.65 | 15.5 |
| PC (18:0/18:2) | 279.3 | | 228.2 | 1.31 | 4.38 |
| | 283.3 | | 200.3 | 1.49 | 4.99 |
| PC (16:0/18:2) | 279.2 | | 338.9 | 0.89 | 2.95 |
| | 255.2 | | 129.2 | 2.32 | 7.74 |

Table XII: LOD and LOQ for PC standards in plasma (Q-Trap analysis)

| PC | product ion in ESI- MRM | concentration (nM) | S/N | LOD (nM) | LOQ (nM) |
|---|---|---|---|---|---|
| PC (16:0/16:0) | 255.1 | 54.13 | 165.7 | 0.98 | 3.27 |
| | 480.3 | 56.06 | 19.3 | 8.71 | 29.05 |
| PC (18:0/18:1) | 281.3 | 50.69 | 57.8 | 2.63 | 8.77 |
| | 283.2 | 51.35 | 27.1 | 5.68 | 18.95 |
| PC (18:0/18:2) | 279.3 | 67.22 | 140.7 | 1.43 | 4.78 |
| | 283.3 | 51.53 | 62.2 | 2.49 | 8.28 |
| PC (16:0/18:2) | 279.2 | 80.01 | 250.4 | 0.96 | 3.2 |
| | 255.2 | 71.87 | 155.4 | 1.39 | 4.62 |

# 4 DISCUSSION

Four phosphatidylcholines were analysed by LC-MS approach. The commercial standards were obtained in a form of powder and they needed to be dissolved prior the analysis. As potential solvents, acetonitrile and dimethylsulfoxid were tested, however, neither of those was suitable, since they did not dissolve PCs properly, even after sonication. Scientifically proven solvent - chloroform (Christie *et* Han, 2012) was avoided due to its toxicity (Nagano *et al.*, 2006). Ethanol was found to be suitable solvent and stock solutions of all PC standards in EtOH were prepared.

The separation of PCs was performed using reversed-phase high performance liquid chromatography with 10 cm long Cortecs C18 column which together with the chosen mobile phases and gradient showed the best separation conditions. However, 5 cm long Kinetex C18 also provided good separation and would be useful for shortening of the analysis time, but the shortening of the analysis time would probably lead to peak overlapping which is not wanted when analysing higher number of phosphatidylcholines. Other columns were not suitable for PC analysis since they did not demonstrate good peak shape or necessary separation. RP-LC provided the distinction of PCs into particular species, based on the interaction of their nonpolar acyl chains with nonpolar stationary phase. The elution order of PCs corresponded to the fact that PCs with higher hydrophobic carbon number (meaning those with longer fatty acyl chains and fewer double bonds) are retained stronger than PCs with lower HCN (Smith *et* Jungalwala, 1981).

The detection of PCs was performed using several mass spectrometric instruments – the instruments being Triple Quad 6410, Triple Quad 6495, QTOF, LTQ and Q-Trap. For reliable quantification of PCs, Triple Quad 6410, Triple Quad 6495 and Q-Trap were the most suitable, since they all have the function of MRM scanning, which allows to set the transition from chosen precursor ion to chosen product ion (Griffiths *et* Wang, 2009). Even though PCs are better ionized in ESI+, the fragmentation in positive ionization did not give any information about PC isobars being present, since all PC species provide the same fragment of phosphocholine head (*m/z* 184 Da) (Taguchi *et al*., 2005). Therefore, it was necessary to perform the analysis in ESI-, since the observed fragments of PCs (when using negative ionization) are the fatty acyl chains and it is possible to distinguish particular PC isobars between each other (Ekroos *et al*., 2003).

As regards other mass spectrometers, LTQ enables the function of $MS^n$ analyses (Thermo Electron Corporation, 2003) which helped to discover the fragmentation behaviour of the studied PCs. All PCs were observed in form of formic acid adducts in ESI-, similarly than with the other mass spectrometers. When using $MS^2$, the acquired fragments were demethylated PCs whereas in $MS^3$, the fatty acyl fragments of PCs were observed (Houjou *et al*., 2004). The analysis of PCs using QTOF was not very successful since it was performed using chromatographic settings and column typical for metabolomics (which showed to be not suitable). However in general, quadrupole-time-of-flight mass spectrometers allow high resolution analyses useful for future global lipidomics experiments (Houjou *et al*., 2005).

If comparing the limits of detection and quantification for chosen PC, the best instrument was Q-Trap (LOD for PC (16:0/16:0 0.97 nM) followed by Triple Quad 6410 (LOD 1.23 nM) and newer Triple Quad 6495 (LOD 5.89 nM). It was expected, that Q-Trap is more selective than Triple Quad 6410 based on advanced technology (Schreiber, 2010). However, from the same reason of improved technology, the new Triple Quad 6495 should have performed better selectivity than Triple Quad 6410 (Agilent Technologies, 2014). The observed lower selectivity of the newer Triple Quad 6495 could be explained by the contamination of the instrument from previous drug analyses or by problems occurring in chromatography.

As regards PC extraction from plasma, 50% MeOH-EtOH (extraction recovery 95.2 % ± 6.2 %) was more efficient extraction solvent for PC extraction, than ACN (extraction recovery 27.5 % ± 10.9 %.). This is related to the fact, that PCs are very well soluble in EtOH (Patil *et al.*, 2010) while in ACN only partly, which was observed during stock solution preparation step. In comparison with other methods of lipid extraction, Cifkova *et al*. (2012) determined the efficiencies of PC extraction being 95 % if using MTBE extraction, 88 % for Folch method and 87 % for Bligh-Dyer method.

We also calculated the matrix effect of plasma which was almost negligible (88.7 % for 50% MeOH-EtOH extraction and 106.2 % for ACN extraction). The matrix effect higher than 100 % (in the case of plasma extracted by ACN) could be caused by ion enhancement (Chambers *et al.*, 2007) or more likely by the deviation of measurement which was about 9.4 %. Relatively low suppression of PCs by plasma matrix can be explained by the fact, that analysed PCs from the class of phospholipids are actually the features, which cause strong suppression of other ions present in plasma sample rather than they are supressed (Chambers *et al.*, 2007) and also by 10 000 times dilution of plasma sample prior the analysis.

Quantification of the four PCs in human plasma was obtained using standard addition method, where a mix of the four PC standards was added in 6 concentrations to diluted plasma and generating a calibration curve from the acquired data. The original PC concentration in the un-spiked plasma was then obtained by extrapolation of "y" from linear regression line to zero response and by multiplication by 10 000 due to the sample dilution. The standard addition method of quantification was chosen, since plasma already contained studied PCs and in addition, it was observed, that each particular PC standard showed different ion intensity in MeOH standard mixture, despite the fact that its concentration in the mixture was identical (PCs with shorter and more unsaturated fatty acyl chains provided higher ionization efficiency than PCs with longer, saturated chains (Koivusalo *et al*, 2001)). For these reasons, the quantifications using internal and external standard were excluded - the quantification by comparison of PCs peak areas to peak area of one internal standard does not take into account the effect of different acyl chains and as regards external standard quantification, it is not possible to secure the same conditions for external standard and plasma samples (standards could not be spiked to the blank plasma since the plasma already contained the analyte of interest) (Yang *et* Han, 2011).

The important fact complicating PC quantification was also high initial concentration of PCs in plasma. It probably led to the formation of lipid aggregates which could not be detected (Yang *et* Han, 2011). Also due to the oversaturation of detector, the nonlinear response was obtained. Therefore, it was necessary to dilute the plasma 10 000 times and also to adjust the concentration range of the spiking solutions, for obtaining a linear calibration curve (final concentrations of 4 PC standards in spiked plasma were 0, 50, 100, 250, 500, 1000 and 2000 nM).

In the Table XIII are shown concentrations of the four PCs in human plasma obtained in our study and PC concentrations measured by Quehenberger *et al*. (2010) in reference plasma (pooled from 100 individuals). It is necessary to note, that Quehenberger *et al*. (2010) did not resolve particular lipid isobars, for example PC 34:2 can theoretically contain PC (16:0/18:2), as well as PC (16:1/18:1) and PC (14:0/20:2).

Table XIII: The comparison of concentrations of the four PCs in human plasma

| PC | detected ion in ESI-MRM | concentration (μM) calculated by standard addition method | PC | concentration (μM) measured by Quehenberger *et al.* (2010) |
|---|---|---|---|---|
| PC (16:0/16:0) | 255.1 | 41.3 | PC (32:0) | 11.4 |
| | 480.3 | 60.6 | | |
| PC (16:0/18:2) | 279.2 | 300.1 | PC (34:2) | 188.0 |
| | 255.2 | 218.7 | | |
| PC (18:0/18:1) | 281.3 | 6.9 | PC (36:1) | 99.8 |
| | 283.2 | 13.5 | | |
| PC (18:0/18:2) | 279.3 | 172.2 | PC (36:2) | 254.0 |
| | 283.3 | 15.3 | | |

The PC values in the Table XIII show, that the concentrations calculated by us and by Quehenberger *et al*. (2010) differ, however the difference could be caused by analysing of different plasma samples, since our sample was taken from one person, while Quehenberger *et al*. (2010) used plasma pooled from 100 people. Also, it is necessary to note, that the calculated concentrations in our study were related to known concentrations of spiking standard solution, and as the PC standards were stored as stock solutions in EtOH which easily evaporates, the real concentration of the standards could be slightly different than we calculated. Therefore, for more reliable quantification, the standard addition method should be done again with fresh standards, which unfortunately were not available for us at the moment.

Regarding the usefulness of phosphatidylcholines as potential biomarkers of AD is still somewhat unclear as in none of the previous studies of Oresic *et al*. (2011), Mapstone *et al.* (2014) and Whiley *et al*. (2014) (Chapter 7.4 Chosen lipidomic studies of AD) were observed really significant differences in levels of chosen PCs between healthy people and AD patients. Therefore further studies are needed to confirm these PCs as representative biomarkers of AD in the future.

# CONCLUSION

Alzheimer's disease is a serious neurodegenerative disease which is still incurable and there are no easily detectable biomarkers, which would help to predict, or reveal the disease in early stage. Since the brain is mostly formed by lipids, it is expected that lipids could play important role in the progression of the disease. The main goal of the thesis was to develop suitable LC-MS method for analysis of chosen lipids in human plasma and to provide their quantification. It was focused on the four lipids from the group of phosphatidylcholines (PC (16:0/16:0), PC (16:0/18:2), PC (18:0/18:1), PC (18:0/18:2)), since they already showed to be changed in plasma of Alzheimer's disease patients. Phosphatidylcholines, in form of commercial standards as well as naturally present molecules in plasma, were analysed by high performance liquid chromatography followed by mass spectrometric detection (using two triple quadrupoles, quadrupole time-of-flight, linear ion trap and quadrupole ion trap), and the final experiments were performed on Q-Trap in MRM mode. Two extraction solvents and several chromatographic columns, mobile phases and gradients were tested to provide the best analysis conditions. The developed LC-MS method for Q-Trap mass spectrometer was able to separate and reliable detect chosen PCs and the quantification of the four PCs in human plasma was obtained and compared with literature. In the future, the key step should be to extend or modify our method for detailed study of chosen lipid features and also to introduce untargeted lipidomics approach for discovery of lipid features which could represent new potential biomarkers of Alzheimer's disease.

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# LIST OF ABBREVIATIONS

AC - acyl carnitine

Acetyl-CoA – acetyl coenzyme A

ACN - acetonitrile

AD - Alzheimer's disease

APCI - atmospheric pressure chemical ionization

APP - amyloid precursor protein

ATP - adenosine triphosphate

CV - coefficient of variation

CID - collision-induced dissociation

CSF - cerebrospinal fluid

Da - dalton

DAG - diacylglycerol

DC - direct current

DNA - deoxyribonucleic acid

ESI - electrospray ionization

EtOH - ethanol

FA - fatty acyl

$FADH_2$ - reduced flavin adenine dinucleotide

GC - gas chromatography

GL - glycerolipids

GP - glycerophospholipids

GPCR - G protein-coupled receptor

HCN - hydrophobic carbon number

HILIC - hydrophilic interaction chromatography

HPLC - high performance liquid chromatography

IP3 - inositol-3-phosphate

IS - internal standard

LC - liquid chromatography

LC-MS - liquid chromatography coupled with mass spectrometry

LIPID MAPS - LIPID Metabolites and Pathways Strategy Consortium

LIT, LTQ - linear quadrupole trap

LOD - limit of detection

LOQ - limit of quantification

*m/z* - mass to charge ratio

MALDI - matrix-assisted laser desorption ionization

MCI - mild cognitive impairment

MDMS-SL - multi-dimensional mass spectrometry-based shotgun lipidomics

MeOH - methanol

MRM - multiple reaction monitoring

MS - mass spectrometry

MS/MS - tandem mass spectrometry

$MS^n$ - multiple stage mass spectrometry

MTBE - methyl-*tert*-butyl ether extraction

$NADH+H^+$ - reduced nicotinamide adenine dinucleotide

NMR - nuclear magnetic resonance

NP-LC - normal-phase liquid chromatography

NLS - neutral loss scan

PC - phosphatidylcholine

PC (16:0/16:0) - 1,2-dihexadecanoyl-*sn*-glycero-3-phosphocholine

PC (16:0/18:2) - 1-hexadecanoyl-2-(10E,12Z-octadecadienoyl)-*sn*-glycero-3-phosphocholine

PC (17:0/17:0) - 1,2-diheptadecanoyl-*sn*-glycero-3-phosphocholine

PC (18:0/18:1) - 1-octadecanoyl-2-(11Z-octadecenoyl)-*sn*-glycero-3-phosphocholine

PC (18:0/18:2) - 1-octadecanoyl-2-(10Z,12Z-octadecadienoyl)-*sn*-glycero-3-phosphocholine

PIP2 - phosphatidylinositol-4,5-biphosphate

PIS - precursor ion scan

PK - polyketides

PKC - phosphokinase C

PLC - phospholipase C

PR - prenol lipids

Q1 - first quadrupole

q2 - second quadrupole

Q3 - third quadrupole

QqQ - triple quadrupole

QTOF - quadrupole time-of-flight

Q-Trap - quadrupole ion trap

RF - radio frequency

RNA - ribonucleic acid

RP-LC - reversed-phase liquid chromatography

SD - standard deviation

SL - saccharolipids

*sn* - stereospecific numbering

S/N - signal to noise ratio

SP - sphingolipids

ST - sterol lipids

TAG - triacylglycerol

TLC - thin layer chromatography

TOF - time-of-flight

UHPLC - ultrahigh performance liquid chromatography

UV - ultraviolet