Qualitative analysis of phospholipids and their oxidized derivatives – used techniques and examples of their applications related to lipidomic research and food analysis

Karol Parchem, Shlomo Sasson, Carla Ferreri & Agnieszka Bartoszek

Accepted author version posted online: 16 Aug 2019.

Submit your article to this journal ↗

Article views: 1

View related articles ↗

View Crossmark data ↗

Qualitative analysis of phospholipids and their oxidized derivatives – used techniques and examples of their applications related to lipidomic research and food analysis

Karol Parchem[1]*, Shlomo Sasson[2], Carla Ferreri[3], Agnieszka Bartoszek[1]

[1]Department of Food Chemistry, Technology and Biotechnology, Faculty of Chemistry, Gdansk University of Technology, 11/12 Gabriela Narutowicza St., 80-233 Gdansk, Poland

[2] Institute for Drug Research, Faculty of Medicine, The Hebrew University of Jerusalem, Jerusalem 9112075, Israel

[3] Istituto per la Sintesi Organica e la Fotoreattività, Consiglio Nazionale delle Ricerche, Via Piero Gobetti 101, 40129 Bologna, Italy

* Corresponding author: Phone: +48 58 347 16 98; fax: +48 58 347 26 94;
e-mail: parchem.karol@gmail.com

Abstract

Phospholipids (PLs) are important biomolecules that not only constitute structural building blocksand scaffoldsofcell and organelle membranes, but also play avital role in cell biochemistry and physiology.Moreover, dietary exogenous PLsare characterized by high nutritional value andother beneficial health effects, which areconfirmed by numerous epidemiological studies.For this reason, PLs are of high interest in*lipidomics* thattargets both the analysis

ofmembrane lipid distribution as well as correlates composition of lipids with their effects on functioning of cells, tissues and organs.Lipidomic assessments follow-up the changes occurring in living organisms, such as free-radical attack and oxidative modifications of the polyunsaturated fatty acids (PUFAs) build in PL structures. Oxidized PLs (oxPLs) can be generated exogenously and supplied to organisms with processed food or formed endogenously as a result of oxidative stress. Cellular and tissue oxPLs can be a biomarker predictive of the development of numerous diseases such as atherosclerosis orneuroinflammation. Therefore, suitable high-throughput analytical techniques, which enable comprehensive analysis of PL molecules in terms of the structure of hydrophilic group, fatty acid (FA) composition and oxidative modifications of FAs, have been currently developed. This review addresses all aspects of PL analysis, includinglipid isolation, chromatographic separation of PL classes and species, as well as their detection. The bioinformatic tools that enable handling of large amount of data generated during lipidomic analysis are also discussed. In addition,imaging techniques such as confocal microscopy and mass spectrometry imagingfor analysis of cellular lipid maps, including membrane PLs,are presented.

Keywords:phospholipids; oxidized phospholipids; lipidomics; LC-MS; bioinformatics; imaging techniques

Abbreviations:1D-LC – one-dimensional liquid chromatography; 2D-LC – two-dimensional liquid chromatography; ANPLC – aqueous normal phase liquid chromatography; CAD – charged aerosol detector; CL – cardiolipins; DAD – diode array detector; DHA – docosahexaenoic acid; DI-MS – direct injection mass spectrometry; DI-SPME – direct immersion solid phase micro-extraction; ELSD – evaporative light scattering detector; EP – ether phospholipid; EPA – eicosapentaenoic acid; ESI – electrospray ionization; FA – fatty acid; GPL – glycerophospholipid; HILIC – hydrophilic interaction liquid chromatography; HPTLC – high-performance thin layer chromatography; IMS – ion mobility spectrometry; LC-MS – liquid chromatography-mass spectrometry; LDL – low-density lipoproteins; LLE – liquid-liquid extraction; LPC – lysophosphatidylcholine; LPE – lysophosphatidylethanolamine; LPL – lysophospholipid; MALDI – matrix-assisted laser desorption/ionization MS – mass spectrometry; MAE – microwave-assisted extraction; MS/MS – tandem mass spectrometry; NMR – nuclear magnetic resonance;

NPLC – normal phase liquid chromatography; oxPL – oxidized phospholipid; PA – glycerophosphate; PC – glycerophosphocholine; PE – glycerophosphoethanolamine; PG – glycerophosphoglycerol; PI glycerophosphoinositol; PL – phospholipid; pPC – phosphatidylcholine plasmalogen; pPE – phosphatidylethanolamine plasmalogen; PPT – protein precipitation; PS – glycerophosphoserine; PSL – phosphosphingolipid; PTLC – preparative thin layer chromatography; PUFA – polyunsaturated fatty acid; RPLC – reverse phase liquid chromatography; ROS – reactive oxygen species; SM – sphingomyelin; SPE – solid phase extraction; TAG – triacylglycerol; TLC – thin layer chromatography; TOF-MS – time of flight mass spectrometry; UAE – ultrasound-assisted extraction;

1. Biological role of cellular, tissue and exogenous PLs and products of their oxidation

Phospholipids (PLs) are amphiphilic molecules composed of lipophilic moiety and a phosphorylated hydrophilic group. They constitute structural building blocks of biological membranes in plant and animal cells and subcellular organelles. PLs also determine biophysical properties of membrane bilayers such as fluidity and permeability

as well as the organization and regulation of the activity of embedded proteins [1]. In addition, membrane PL-derived polyunsaturated fatty acids (PUFAs) are precursors of numerous signaling molecules such as eicosanoids, leukotrienes, prostaglandins and thromboxanes that are important modulators of both inflammation

and anti-inflammatory signals [2].

Dietary PLs are known for their high nutritional value and beneficial health effects. Numerous epidemiological studies indicate that foodborne PLs may prevent or modulate the progression of chronic diseases. Dietary PLs and products of their enzymatic metabolism may affect pathways related to inflammation and regulate lipid metabolism [3]. PLs also normalize blood lipid profiles by reducing the absorption of cholesterol, triacylglycerols (TAGs) and fatty acids (FAs) in the intestine. Therefore, dietary PLs, especially PUFA containing are thought to reduce the risk of cardiovascular diseases [3]. Consequently, some sources, such as krill oil, are considered nutritionally beneficial due to the high abundance of n-3 PUFAs (i.e., eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) in the PL structure [4–6]. Moreover, previous studies demonstrated that PUFAs delivered in PL structure are more efficiently incorporated into cell membranes than these present in TAGs [7]. This is probably

because PUFAs build in TAG structure are preferentially used as the energy source for skeletal muscles and heart cells or are directed to adipocytes [8]. For instance, it was found that dietary PLs are needed for the regeneration of damaged hepatocytes and proper function of nervous system [1].

Yet, PUFAs within PLs are particularly susceptible to oxidation during manufacturing, storage and final preparation of foods (e.g., frying, baking or grilling) [9]. Previous studies indicated that dietary oxidized PLs (oxPLs) can be toxic to epithelial cells of digestive tract, which are directly exposed them. The accumulation of dietary oxPLs by the digestive tract cells may also induce oxidative stress that can cause cell membrane oxidation and DNA or protein damage leading to some gastrointestinal pathologies [10]. In addition, dietary oxidized FAs build in PL and TAG structure, after intestinal digestion and absorption by enterocytes, can be released into the portal vein and circulatory system. These oxidized lipids may contribute to the etiology of peripheral diseases, such as atherosclerosis [11,12].

On the other hand, PUFAs within cellular PLs are susceptible to endogenous oxidation upon cellular oxidative stress. Such endogenous oxPLs can be involved in two interrelated processes: first, cell and tissue damage and second, activation of signalling pathways that mediate inflammatory response and apoptosis [13]. There is an ample evidence that both ingested and endogenously formed oxPLs are also integrated in oxidized low-density lipoproteins (LDL), which further contribute to the development of cardiovascular diseases including atherosclerosis, thrombosis and coronary artery disease [14,15]. Although, oxPLs have been implicated in the development of numerous disorders, they occur not only in pathological states but also under healthy conditions. The difference between pathological and physiological conditions seems to be quantitative, the elevated levels of oxPLs species were identified in various tissues or blood. Therefore, oxPLs have been proposed as potential biomarkers in breast cancer [16], hyperlipidemia [17] neuroinflammation [18], lung injury [19] or alcoholic patients [20], though none of the oxPLs have yet been validated.

2. The development of lipidomics and related sciences

Recent advances in research on function of lipids in cells, tissues, organs and whole organism under both physiological and pathological states contributed to the development of *lipidomics* – an interdisciplinary subfield of metabolomics that focuses on the global study of

the lipidome, which is defined as the collection of lipid compounds within a given biological system [21]. Lipidomic studies embrace the identification of individual lipid species, subcellular localization and tissue distribution as well as changes in their abundance and concentration. Such knowledge is necessary to enable the discovery of molecular mechanisms of lipid action under physiological conditions as well as during the development of disorders. One of the branches of lipidomics is *membrane lipidomics* which nowadays has been attracting growing interest because the membrane compartment is a highly organized system and the choice of PLs reflects biophysical rules as well as biochemical and physiological outcomes of the whole cell [22,23]. In parallel, the oxidative reactivity of the PLs can affect membrane organization thus cellular and homeostasis, including the natural process of membrane PL replacement [24]. Therefore, efforts have been undertaken to clarify the interplay between PL organization and reactivity to contribute to the full comprehension of the cell management. Nevertheless, it can be stated that *oxidative lipidomics* does not develop as fast as lipidomics. The non-increasing annual number of publications with keywords "*oxidized lipids*" and "*oxidized phospholipids*" can confirms this observation (**Figure 1**)[25]. On the other hand, since the 90s of the last century, a growing number of publications under "*lipid oxidation*" keyword have been observed (**Figure 1**). The explanation of this phenomenon may be fact that "*lipid oxidation*" *per se* is a very general term that has been used in various areas of research to determine the impact of free radicals on lipids present in clinical samples such as fluids and tissues (redox biology) or foodstuffs (food quality). The lipid oxidation reaction in these studies is often monitored by simple and unspecific spectrophotometric methods, such as TBARS assay. Whereas screening, identification and characterization of oxidised lipids and PLs during oxidative lipidomic studies requires the use of advanced and not so affordable methods.

The results of epidemiological studies indicate that the type, quality and intake of dietary lipids may contribute to the prevention or promotion of diet related metabolic diseases including obesity, type 2 diabetes, atherosclerosis or hypertension. Therefore, lipidomics and membrane lipidomics are considered powerful tools to investigate the interactions between diet, nutrients, genes and human metabolism, both in physiological and pathological conditions [21]. This approach contributed to the introduction of *foodomics* – a discipline that investigates the food and nutrition domains through the application of advanced *omics* technologies to improve wellbeing, human health, and consumer knowledge [26,27]. The foodomic studies involve

not only metabolomics, including lipidomics, but also genomics, epigenomics, transcriptomics and proteomics for compound profiling/authenticity and/or biomarkers analysis related to food quality or safety [27,28].

In general, two analytical strategies can be distinguished in the lipidomic studies. Targeted analyses focus on the determination of a set of known lipid species. However, it should be noted that modern methodologies enable large-scale lipid profiling, embracing hundreds of compounds [29]. The second strategy is thus the untargeted analyses that allow a more comprehensive evaluation of lipidomic profile. In this approach, the entire data sets comprising thousands of metabolite signals are processed, which enables detection and identification of unknown lipids as well. Some of them can be identified as potential discriminators of different physiological or pathological states. However, it should be noted that the targeted analyses provide more sensitive and accurate detection of lipids, which is the consequence of monitoring of predetermined compounds for which the analytical methodology was developed [29].

The development of lipidomics has been advanced in the last decades for the availability of analytical techniques and proper instruments that enable qualitative and quantitative analysis of lipid compounds in complex matrices, such as biological and food specimens. The application of mass spectrometry (MS) with soft ionization techniques was the key step towards the advent of lipidomics. The first report on the application of electrospray ionization (ESI) [30] and matrix-assisted laser desorption/ionization (MALDI) [31] for the analysis of complex PL mixtures was published in 1994 and 1995, respectively. Since then, applications of shotgun (direct injection) MS and liquid chromatography coupled with MS (LC-MS) have become dominant techniques in lipid analysis, including PLs. Additionally, other techniques such as gas chromatography (GC), thin layer chromatography (TLC) and nuclear magnetic resonance (NMR), especially ^{31}P NMR in analysis of PLs [32], are widely applied in lipidomic analysis [33]. It must be immediately clarified that so far PLs containing geometrical and positional isomers cannot be satisfactorily analysed to distinguish molecules with the same mass due to the complex mixtures of lipids present especially in biological samples. GC and further fatty acid derivatization procedures using dimethyl disulfide or other reagents are necessary to explore the complex isomerism of fatty acid structures [34].

In order to summarize the current state of knowledge concerning the comprehensive analysis of PL species and their oxidized derivatives, we reviewed the analytical techniques

used in lipidomics and related sciences. This review covers all qualitative aspects of PL analysis, from the lipid isolation through the chromatographic separation of PL classes and species to their detection. We discussed standard techniques used for PL analysis, which can be successfully applied in most laboratories as well as advanced approaches such as two-dimensional chromatography coupled with MS or ion mobility spectrometry (IMS) that enable more detailed characterization of complex lipid samples. We presented also bioinformatic tools applied for handling large amount of data generated during MS-based analysis. Moreover, confocal microscopy for imaging of cellular lipids, using various fluorescence probes as well as mass imaging techniques are presented and discussed in this review.

3. Occurrence and structural diversity of PL and generation of their oxidative derivatives

According to the backbone structure, PLs are classified in two major groups: glycerophospholipids (GPLs) and phosphosphingolipids (PSLs) (**Figure 2**)[35]. Lipophilic fragment of GPLs consists of two FAs linked to L-glycerol by ester bonds in the *sn*-1 and *sn*-2 positions forming diacyl moieties [36]. Naturally occurring plant and animal GPLs typically contain a saturated FA in the *sn*-1 position and an unsaturated FA in the *sn*-2 position [1]. In the case of ether phospholipids (EPs) (present in animal and microbial organisms), FA in the *sn*-1 position is replaced by long-chain O-alkyl or O-alkenyl groups [37]. The core structure in PSLs is a long-chain amino alcohol – sphingoid base. Depending on the origin, sphingoid bases differ in their alkyl chain length (12 – 22 carbon atoms), degree of unsaturation and positions of double bonds. The most abundant sphingoid base found in animal tissues is the 18-carbon sphingosine (2-amino-4-octadecene-1,3-diol), which is linked via amide bond to FA, most frequently saturated. This amides are termed ceramides [36]. In plants, the common sphingoid base is phytosphingosine (2-amino octadecano-1,3,4-triol) or its unsaturated analogues [38].

The polar moiety of GPLs, EPs and PSLs includes an orthophosphate group forming a phosphodiester bond between a hydroxyl group of the backbone (present in the *sn*-3 position of glycerol or linked with the first carbon atom of the sphingoid base) and hydroxyl group of hydrophilic group. The orto-phosphate group can be replaced by phosphono group, which is found in the structure of glycerolophosphonolipids and phosphonosphingolipids (**Figure 2**). In this case, the carbon atom of the hydrophilic group is linked directly with phosphorus atom in phosphono group [39].

Based upon the structure of the hydrophilic group, PLs are categorised into several classes: glycerophosphocholines (PCs), glycerophosphoethanolamines (PEs), glycerophosphoserines (PSs), glycerophosphoinositols (PIs), glycerophosphoglycerols (PGs), glycerophosphoglycerophosphoglycerols (also known as cardiolipins (CLs)) and glycerophosphates (PAs) [35]. In addition, as a result of the activity of plant or animal phospholipase A[1] and A[2], GPLs can be hydrolysed respectively in position *sn*-1 or *sn*-2 of glycerol to form lysophospholipids (LPLs) such as lysophosphatidylcholines (LPCs) and lysophosphatidylethanolamines (LPEs) [36]. The most abundant class of PSLs are sphingomyelins (SMs), in which choline is the hydrophilic group [36]. In nature, ceramide derivatives of ethanolamine and inositol are also found [38].

The PL fraction is often called lecithin, but this term does not define chemical structure. This term is used to describe PLs of plant (e.g., rapeseed or soybean lecithin) or animal (e.g., egg yolk lecithin) origin [40]. Moreover, in lipid technology this name is often used for PL fraction dissolved in ethanol, whose main component is the PC fraction. The fraction insoluble in ethanol is named cephalins, and mainly contains the PE fraction.

PUFAs, such as linolenic acid, arachidonic acid, EPA and DHA, in PLs can undergo oxidation. Oxidized PLs (oxPLs) are generated in biological systems as a result of enzymatic oxidation and/or random free radical-mediated non-enzymatic peroxidation [41,42]. Cellular PLs can be modified directly by specific enzymes such as lipoxygenases, cyclooxygenases or cytochrome c [43]. The peroxidation of PLs is also induced by direct interactions with reactive oxygen species (ROS) and reactive nitrogen species (RNS), which either penetrate cells or are generated endogenously by cellular processes (e.g., mitochondrial origin or NADPH oxidase) [19]. In turn, dietary oxPLs are formed during food production as a result of non-enzymatic autooxidation and photooxidation or enzymatic oxidation catalysed by lipoxygenases [9].

The structure of the final oxidation products generated via radical-based peroxidation depends on several factors including the nature of oxidant species, the unsaturation degree of FA linked to the glycerol backbone and the positions of double bonds [44]. The abstraction of hydrogen atoms located on methylene groups of unsaturated FA, and subsequent reaction with oxygen, leads to the formation of peroxy radical. In the next step, this radical can initiate a next peroxidation reaction with another unsaturated FA and finally is stabilized by PL hydroperoxide formation. These primary oxidation products (PL hydroperoxides) are

characterized by low stability and can decompose via monomolecular or bimolecular reactions [9] to long-chain oxPLs containing hydroxy, polyhydroxy, epoxy or keto groups [45]. The long-chain oxidation products further undergo oxidative changes that lead to the formation of short-chain oxPLs containing saturated and unsaturated aldehydes, hydroxy-aldehydes, carboxylic acids and hydroxy carboxylic acids as well as furan derivatives [44]. In addition, volatile low molecular weight compounds such as pentanal or hexanal are formed as a result of C-C bond cleavage [45].

4. Sample preparation

The first stage of lipidomic studies requires the isolation of lipid fractions from matrices in which these molecules are usually strongly embedded. This process aims also at separation of non-lipid substances such as proteins, sugars, and low molecular weight compounds that could interfere with further steps of the analysis [46]. We believe that the standardization of the methods for lipid isolation is still an unaddressed problem, which influence the use of the data for meta-analysis. Lipidomic applications require high yield of analyte isolation, reproducibility, but also short preparation time. In addition, the final extracts should be provided in a forms suitable to the instrumental techniques used in the subsequent steps of analysis [33]. For example, shotgun lipidomics, where lipid species are analyzed by MS and/or tandem MS after direct injection, is highly dependent on the extract quality, since any non-lipid substances and water contamination could led to significant ion suppression and/or high chemical noise. Consequently, this can result in reduced sensitivity and inaccurate or irreproducible MS measurements [47]. The techniques of initial sample preparation for PL analysis include liquid-liquid extraction (LLE), protein precipitation (PPT) and, more recent, solvent saving direct immersion solid phase micro-extraction (DI-SPME), which has attracted considerable attention [33,48,49]. Some enrichment of lipid fraction in PLs is possible with the aid of solid phase extraction (SPE) procedures. Sample preparation techniques used in the PL analysis, examples of their application and information on the required sample volumes, isolation time, cost, recoveries, lipid coverage, repeatability/reproducibility and potential for their automation are presented in **Table 1**.

4.1. Sample collection and storage

PLs and especially their oxidized derivatives are very unstable compounds; therefore sampling and storage conditions are key factors affecting sample quality. Since lipid degradation, for example resulting from uncontrolled oxidation or hydrolysis, can occur within a few seconds, the material should be protected as quickly as possible. The preparation of analysed material depends mainly on the origin and form of the sample. The isolation of lipid fractions from plant, animal and human tissues as well as solid food samples requires usually quick freezing in liquid nitrogen and grinding before the addition of organic solvents [65,66]. Samples derived from cell cultures must be first thoroughly washed from serum-borne lipids. Depending on the further procedure used, the cells can be homogenized [48] or more commonly the lipids are extracted from the cell pellet directly by the addition of cold solvents [65,67]. In turn, liquid samples, such as serum or plasma as well as foodstuffs, e.g., milk, can be extracted directly using cold solvents or following freezing and storage [66,68]. The non-enzymatic lipid peroxidation may occur even at -20 °C. Therefore, if immediate extraction is not possible such samples should be stored at -80 ° C for a few months. Moreover, to avoid oxidative modifications of lipids, the extraction solvent systems are commonly enriched with antioxidants, among which butylated hydroxytoluene (BHT) is most frequently added [66]. However, it should be noted that antioxidants may reduce oxidized lipid species, and therefore they should not be added to samples in which oxPLs are to be analysed [69]. Instead, other protectors, such as metal chelators (e.g., EDTA, EGTA or DPTA) are used along with low temperature, nitrogen atmosphere and protection from light [46,66].

Sometimes, there is a necessity to thaw the samples several times to perform analysis of various parameters. Hammad et al. determined the effect of freeze and thaw cycles on the stability of SM(C16:0) in human blood serum and plasma samples. The results showed that the first and second freeze and thaw cycle do not affect the level of this SM specie, while a statistically significant change was observed after the third cycle [70]. Therefore, a better solution may be to divide samples before freezing into smaller single-use portions to avoid multiple freeze and thaw cycles, which will ensure high sample quality for all planned analyses. Some authors have also determined the effect of the anticoagulant type used for preparation of plasma samples on PL concentration. The results obtained by Gonzalez-Covarrubias et al. indicated that among 80 endogenous lipids, the content of 71 species significantly depended on two or all tested anticoagulants (sodium citrate, potassium EDTA and lithium heparin)

[71]. Statistically significant differences between two types of anticoagulants on PL recovery were observed for the majority of PLs including LPC, LPE, PC, PE and SM species, while variations among all three tested anticoagulants were found for LPC(16:0), PC(40:4), PE(36:3), SM(d18:1/16:0) and plasmalogen form pPE (38:5), pPE (36:5). Most importantly, for all PL species (only except PC(40:2)), the extraction efficiency was highest in the case of potassium EDTA [71].

During sampling of human or animal biological material such as blood, special should be also paid to the effect of circadian cycle on the variability in composition of individual lipid species, including PLs. The human studies involving 20 healthy males identified in some of them, the circadian-regulated PC species in the plasma [72]. For example, the peak concentration levels in the evening was observed for PC(32:2) and plasmalogen forms such as pPC(34:0), pPC(34:1), pPC(34:2) and pPC(36:2). In addition, circadian variation in LPC(16:0) and SM(d18:1/18:1) levels were found. The studies carried out by Kasukawa et al. also showed diurnal oscillations in LPC(16:0) as well as PG(18:1/0:0) levels [73]. In turn, Aviram et al. indicated changes in nuclear and mitochondrial lipidome in mouse liver throughout the day and upon different feeding regimens [74]. In the mentioned cell organelles, 222 lipid species belonging to different PL classes such as CLs, lysocardiolipins (LCLs), LPEs, LPEs, PAs, PCs, PEs, PGs, PIs, PSs and SMs were identified and quantified. Among 152 lipids found in the nucleus and 217 lipids in mitochondria, 34% and 31% of lipids, respectively, showed diurnal oscillations. In both organelles, predominant oscillating lipids were PC and PE species. In addition, the results indicated that these oscillations in nucleus and mitochondria are anti-phasic. Analysis of the peak accumulation time for the various oscillating PLs showed that the vast majority of nuclear lipids peaked early in the light phase at zeitgeber time. In contrast, most diurnal-regulated mitochondrial PLs reached their maximum levels at the transition from the light to the dark phase [74].

Another important factor that can affect the lipid profile is food intake. As already mentioned, Aviram et al. determined various feeding regimens and showed that there is a significant difference between mice fed *ad libitum* (described above) and exclusively during the night. In the case of nucleus lipidome, the night-restricted feeding led to an increase in the fraction of oscillating PLs, whereas in mitochondria, its moderate decrease was observed. Moreover, under night-restricted feeding, the vast majority of oscillating PLs accumulated in the

nucleus at the beginning of dark phase, whereas in mitochondria PLs peaked at the beginning of light phase [74]. This contrasted with the PL fluctuation observed for *ad libitum* fed mice, thus showed a significant effect of feeding regimens on lipidome of individual cell organelles.

In sample preparation an interesting observation can be made in the case of the red blood cell (RBC) membrane lipidomic analysis, since it is well known that the lifetime of these cells is around 4 months. During this period, the RBCs come into contact with all the body tissues. Consequently, their membrane PLs can carry an important information related to nutritional habits and metabolism. The isolation of mature RBCs, whose characteristics (such as density gradient and cell diameter) is well known, can be nowadays carried out by robotics, thus avoiding tedious and imprecise manipulations [75]. Generally speaking, technical and methodological aspects, especially in the case of the preparation of biological samples such as RBCs, should be thoroughly discussed among the scientific community, working toward unification and standardization of the procedures. This would be very important for improving the analytical efficiency and establishing calibrated parameters for metabolic evaluation.

4.2. Isolation of PL fractions

LLE is the most commonly applied extraction procedure, appropriate for a wide range of apolar and polar lipid compounds, including PLs. Best known is the Folch's method that was introduced in 1957. In this protocol, chloroform/methanol (2:1 v/v) extraction mixture is employed followed by washing the extract with saline solution (0.9 % NaCl or KCl) [76]. The main drawback of Folch's method is the substantial consumption of organic solvents, as it requires a 20-fold excess of chloroform/methanol mixture over the sample volume. Therefore, several alternative approaches were developed, among which the most commonly used is Bligh and Dyer method [77]. The extraction yield of these two methods is similar for samples containing less than 2 % *w/w* lipid fraction. However, for samples richer in lipid content, the recovery of total lipid fraction by the Bligh and Dyer method can be even 50 % lower than in the Folch's procedure [78]. The disadvantage of both methods is the use of a toxic solvent – chloroform. Therefore, in some studies, the less toxic replacement – dichloromethane, which provides similar extraction efficiency as the Folch's method, is used [79]. Nevertheless, in order to avoid toxic solvents, other extraction methods have gained popularity in lipidomic studies in recent years. An example of such approach is the method introduced in 2008, which employs the mixture of methyl tert-butyl ether (MTBE) and methanol (1.5:1 *v/v*) followed by

washing the extract with water added at a 5:1 *v/v* ratio [80]. In contrast to chloroform and dichloromethane, MTBE organic phase is lighter than water and constitutes the upper layer in this two-phase system. However, the MTBE extract contains quite a large amount of water, which requires a longer time to evaporate the solvent under a stream of nitrogen and results in more sodium adducts in comparison with extraction using chloroform along with possible ion suppression and/or high chemical noise (especially in the case of shotgun MS) [47]. Another chloroform-free protocol for the lipidomic approach is the butanol-methanol (BUME) method. This protocol includes an initial extraction of lipids using butanol/methanol mixture (3:1 v/ v) followed by extraction using heptane/ethyl acetate (3:1 v/v). Then, the phase separation is induced by addition of 1 % aqueous acetic acid [56]. Initially, the BUME method was used to isolate lipids from plasma samples, but then it was developed to extend the possibilities of its use in variety biological samples such as tissues and cultured cells [47]. Both mentioned chloroform-free methods are characterised by comparable lipid extraction efficiency, while the organic phase of the BUME method contains less water than organic phase in the case of MTBE method [56]. In addition, it should be noted that both these method (MTBE and BUME) were developed to their suitability for automation of PL and other lipid analysis. Another extraction solvent system proposed to isolate lipids from biological fluids and tissues is hexane/2-propanol mixture (3:2 *v/v*). Plasma can serve as an example of application of this method. In this case, the solvent mixture is added to the sample in a ratio of 8:1, followed by vigorous mixing and transfer to an ultrasound bath for 5 min. Then, an aqueous solution of Na_2SO_4 is added, the upper hexane phase is transferred to new tube and the bottom phase is re-extracted with a new portion of hexane/2-propanol mixture (3:2 *v/v*). After pooling both extract portions, the solvent is evaporated [81].

The extraction efficiency of individual lipid classes, including PLs and LPLs, using most of the above-described methods has been tested on the example of human LDL (**Figure 3**) [51]. The LC-MS/MS technique was employed for qualitative and quantitative analysis of isolated lipids using five different solvent extraction protocols: Folch, Bligh and Dyer, acidified Bligh and Dyer, MTBE/methanol and hexane/2-propanol. The qualitative analysis enabled to identify more than 350 different lipid species belonging to 19 subclasses. It was found that the solvent composition had a small effect on the extraction of major lipid classes, such as TAGs and cholesterol esters, while the extraction of less abundant lipids was strongly dependent on

the solvent system used (**Figure 3**). Based on these results, it can be stated that the acidified Bligh and Dyer protocol was the most efficient method for extraction of PLs including, PCs, SMs, PEs and LPCs, while Folch's method allowed the highest recovery of PIs [51].

In addition to the classic lipid extraction methods described above, alternative techniques are used to shorten the extraction time and to reduce solvent consumption. Among them microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE) can be distinguished, wherein the latter is more commonly used for the lipid extraction from biological and food samples [60]. In the case of MAE, microwave energy is used to increase temperature and pressure during extraction. However, it should be noted that the generated heat could lead to the degradation of thermally labile lipids. For this reason, some authors suggest that MEA parameters require careful optimization to allow high extraction efficiency of targeted lipid analytes. For example, it has been found the temperature of 60 °C and time of 10 min were optimal conditions during extraction of yeast lipids, including various PL classes (PAs, CLs, PEs, SMs, PSs and PIs) [61]. In turn, ultrasonic energy used during UAE facilitates mass transfer between two immiscible phases, which increases extraction efficiency and reproducibility. The studies carried out by Pizarro and co-workers have shown that combination of MTBE-based extraction method with UAE technique resulted in the detection of 30 % more lipid species in human-blood samples compared to classical LLE method with MTBE [59]. Moreover, the advantage of UAE technique is the ability to control the temperature during extraction. For example, low temperature of the samples during extraction can be simply maintained by an ice bath, which makes this technique attractive for thermally labile lipids.

Another sample preparation strategy for lipidomic studies, which is widely used for biological fluids such as blood plasma [62,82] involves protein removal by precipitation. This technique is also useful in the analysis of metabolites occurring in food samples, such as cow and goat milk [83]. This approach involves protein removing from the samples by the addition of water-miscible solvent such as acetonitrile, methanol, ethanol or 2-propanol, which in contrast to LLE technique leads to the formation of monophasic system. If the concentration of analytes in the sample is high enough for the detection method used, the sample can be directly injected after protein precipitation (PPT) [65]. Sarafia et al. compared four PPT methods using different water-miscible solvents (methanol, acetonitrile, 2-propanol and mixture of 2-propanol/acetonitrile (1:2 *v/v*)) with LLE methods employing four different solvent systems

(methanol combined with chloroform, dichloromethane or MTBE as well as isopropanol with hexane). The results indicated that protein removal was more efficient using PPT technique than LLE. Especially, 2-propanol appeared to be the most suitable solvent for human blood plasma enabling a broad coverage and recovery of lipids, including PC, PE, PS, PG, LPC and SM classes [62]. Another report showed that recovery values for PC, PE and PI species were almost equivalent for methanol, ethanol and 2-propanol. Slightly higher recoveries of some LPC species were observed for methanol, which also proved to be the best for all tested LPI species [84].

Apolar lipids such as TAGs, cholesterol and its esters, which are present in fractions obtained following total lipid extraction or protein precipitation, can interfere with further steps of PLs analysis. Therefore, apolar lipids can be selectively removed using SPE technique, in which columns containing silica gel as stationary phase are commonly used. Initially, apolar lipids are eluted with hexane and diethyl ether, while PLs that remain bound to stationary phase are subsequently eluted with methanol followed by the mixture of chloroform, methanol and water. This approach allows almost a complete recovery of the PLs [68,85].

To best of our knowledge, there are no specific methods or protocols for selective isolation of oxPLs from biological and food samples. Currently, the sample preparation prior to the analysis of PLs modified by oxidation includes conventional techniques such as LLE [66,86,87] and PPT [63]. Commonly, chloroform-based methods are still preferred in the case of oxPLs extraction, most likely due to the effective isolation of a wide range of lipids, including oxidized derivatives [25]. The influence of additional factors such as ultrasound or microwave treatment on the extraction efficiency of oxidized lipids has not yet been determined. Therefore, due to the low concentration level of oxPLs in total lipid extract obtained by means of LEE or PPT techniques, the pre-concentration of samples may be required. Alternatively, the separation of the oxidized species from unoxidized and neutral lipids present in the sample can be achieved already at the sample preparation stage. Both of these challenges can be overcome using SPE technique. For example, this technique was used to targeted analysis and quantification of plasma oxPL species recognized as molecules with potential diagnostic value in acute inflammation, cardiovascular disease and atherosclerosis [63]. For this purpose, the micro-SPE (μSPE) method employing C18-modified silica gel spin-columns was tested. After loading the lipid extract, the columns were washed with methanol/0.2 % formic acid (70/30 *v/*

v) to remove salts and majority of hydrophilic species such as LPCs. Then, oxPCs were mainly eluted with methanol/0.2 % formic acid (82:18 *v/v*), while non-oxidized PLs were subsequently eluted with methanol/0.2% formic acid (98:2 *v/v*). Finally, 2-propanol washout removed residual lipids such as TAGs, after the reconstituted columns were ready again for further use. The recovery of oxPC species in the fraction enriched with oxidized derivatives was around 70-75% [63,64]. Another tested strategy of oxPL enrichment consisted in the application of surface functionalized nanoparticles [88]. This method involves a derivatization with a bifunctional reagent containing both a hydrazide group for hydrazone formation with aldehyde-containing oxPLs and a thiol moiety enabling subsequent trapping with nanoparticles. After washing step, the trapped analytes are quantitatively released from the nanoparticle surface as a result of transimination with hydroxylamine. Then the released oxime-derivatives of the carbonyl-containing oxPLs are analyzed. So far, this approach was applied to prepare enriched extract of oxPCs from samples such as human plasma with recoveries up to 90 % [88].

The conventional sample preparation methods, LLE and combined liquid-liquid extraction and solid phase extraction (LLE-SPE) are based on manual lipid isolation. These methods are overly time consuming to be employed for routine analyses of many samples, which is commonly required in lipidomic studies [48]. Therefore, there has been recently a growing interest in direct immersion solid phase micro-extraction (DI-SPME) for the study of lipid compounds, including PLs. DI-SPME technique is based on adsorption of analytes on the surface of thin glass or quartz fiber coated with a specific sorbent. Silica-based sorbents modified with C18 groups are often used for lipidomic applications [48,49,89]. When the analyte concentrations reaches distribution equilibrium between the fibres surface and the sample matrix, the coated fibers are immersed in purified water to remove sample residues. Then, lipid compounds are desorbed by immersion of the fibers in organic or aqueous organic solvent [48,49]. The prepared extract can be then analysed using LC-MS or shotgun MS. Current DI-SPME devices are available with a fully automated 96-well plate systems that allow simultaneous extractions of many samples at the same time [48]. Among the advantages of the DI-SPME technique are the shorter sample preparation time, lower use of organic solvents (especially toxic chloroform) and smaller sample volume, which is particularly important for scarce material such as clinical samples [49].

To summarize this chapter, special attention should be paid to the fact that sample preparation is on the one hand very important to generate high quality lipidomic data sets. On the other hand, it should be noted that there is no universal method for isolation of PLs and their oxidized derivatives from different sources. The Folch's, Bligh-Dyer and Matyash extraction methods were originally developed to the specific matrices, such as brain tissue, fish muscle, *Escherichia coli*, and their direct application to other types of samples may not provide satisfactory extraction efficiency and lipid coverage. For this reason, it seems necessary to optimize the isolation condition, such as solvent system, the ratio of the sample amount to the solvent volume or the effect of additional factors such as ultrasounds. Particular care must be taken during optimizing the isolation of oxPLs that are characterized by relatively high polarity and may be lost upon standard procedures dedicated to lipid isolation.

5. Separation of PL

5.1. Thin layer chromatography

Thin layer chromatography (TLC) is a relatively simple and inexpensive technique [90] for quick separation of different PL classes. This technique can provide preliminary information about PL composition and the presence of oxidized species (e.g., PL hydroperoxides), thereby helping to properly choose more advanced analytical approaches. The silica gel coated high performance TLC (HPTLC) plates are most commonly employed in PL analysis, which allows not only separation of individual PL classes, such as PAs, PCs, PEs, PIs, but also products of their hydrolysis, including LPAs, LPCs, LPEs and LPIs [91].

In contrast to analytical HPTLC, preparative TLC is characterized by high capacity and allows separation of larger amounts of analytes (10 – 1000 mg) [92]. Therefore, the latter technique can be applied to isolation of individual PL classes for further analysis, e.g. using GC. After chromatographic separation, the compounds of interest can be extracted from the respective areas of the preparative plate with the aid of organic solvents, e.g., chloroform/ methanol mixture (1:2 *v/v*) [93].

The main components of the mobile phase used in one-dimensional TLC (1D TLC) separation of PL classes are chloroform, methanol and water. The mobile phase composition can be adjusted depending on the variety of PL classes present in the samples by adding organic solvents (e.g., acetone, methyl acetate, 2-propanol, triethylamine, ethanol) and modifiers (e.g.,

acetic acid, ammonia) [91]. An example of commonly used 1D TLC system that allows the analysis of samples with a diversified composition of PL classes is a mixture of chloroform, methanol, acetic acid, acetone and water (35:25:4:14:2 *v/v/v/v/v*). This solvent system enables separation of LPCs, SMs, PCs, LPIs, LPEs, PSs, PIs, PEs, PAs, PGs and CLs [93,94].In our research, we employed this chromatographic system to determine composition of PL classes in both biological samples (lipid fraction isolated from HT-29 cells) and food product (soy lecithin) (**Figure 4**). However it should be indicated that native and oxidized species belonging to the same PL class tend to co-elute during 1D TLC separation using silica gel coated plates. Nevertheless, the detection of oxPLs among their native counterparts is possible by means of specific derivatizing reagents, as described at the end of the section.

For very complex PL composition, it is recommended to use two-dimensional TLC (2D TLC) with higher resolution capability. In the first dimension, PL classes are separated using solvent mixture with higher elution strength. A typical mobile phase used at this stage consists of chloroform, methanol and 28% ammonia solution (60:35:8 *v/v/v*). After chromatogram development and drying of the plate, PL classes are further separated in the second dimension at a 90-degree angle from the first run. In this case, the mobile phase with lower elution strength (e.g., chloroform, acetone, methanol, acetic acid and water (50:20:10:10:5 *v/v/v/v/v*)) is employed [95,96]. Despite the significant improvement in chromatographic resolution, a major disadvantage of 2D TLC is the limit of one sample analysis per plate, whereas nearly two dozen of samples can be applied in a single 1D TLC run [90].

The key factor to achieve high resolution and reproducibility are the experimental conditions in developing chamber. Therefore, it is recommended that the chamber should be homogenously saturated for given durations (at least 20 min). Similarly, humidity must be controlled to maintain adequate stationary phase activity, which plays an important role in the separation of PL classes on the silica plate [91]. These combinations can be fulfilled with the use of automatic developing chambers e.g., delivered by CAMAG, Switzerland.

Once PL separation by means of TLC technique, the next step is their detection with non-specific or specific derivatizing agents. Among the latter, PL specific reagents [97] or reagents interact with individual hydrophilic groups of PLs [93], such as choline, ethanolamine or serine, can be distinguished (**Table 2**). TLC technique may also be useful for the detection and identification of oxPLs such as hydroperoxide derivatives. The presence of PL

hydroperoxides, e.g., in heat-treated food samples or oxidatively challenged cultured cells, can be detected following spraying the plate with a solution of dimethyl-*p*-phenylenediamine (DMP) or tetramethyl-*p*-phenylenediamine, which results in purple staining of oxidized moieties[69,98]. For example, in our research, we applied HPTLC technique with DMP-visualization to monitor the kinetics of lipoxygenase-catalysed peroxidation of egg yolk phospholipid fraction (**Figure 5**) [99]. The HPTLC profiles revealed the differences in the rate of PL hydroperoxides formation and degradation between PC and PE classes caused by the varied composition of FAs in their structures.

It is important to limit the sample exposure to atmospheric oxygen throughout the oxPLs analysis in order to avoid uncontrolled oxidation [69]. Our experience indicates that the most sensitive step in which uncontrolled PL oxidation can occur is application of samples onto TLC plates. Therefore, it is recommended to apply the samples using manual or automatic TLC applicator fitted with a nitrogen cover shield [69,98].

5.2. Liquid chromatography

The most widely analytical tool used for the identification and quantitative analysis of PLs present in biological, pharmaceutical or food samples is liquid chromatography (LC). The employment of normal phase LC (NPLC) and hydrophilic interaction liquid chromatography (HILIC) enables the separation of individual PL classes according to their hydrophilic group structure [41,46,68,106]. In turn reverse phase LC (RPLC) ensures the separation of PL species belonging to the same class but differ in their FA composition. Individual PL classes can be separated in terms of the length and unsaturation degree of FAs, and also by the type and degree of oxidative modification of FAs present within the PL structure [41,46,106–108].

5.2.1. Separation of PLs into classes

The structure and chemical properties of PLs enables their separation into individual classes by means of NPLC. For this purpose, columns with a length of 100 − 250 mm packed with unmodified silica gel [41,93] or modified silica gel with diol groups [109,110] with grain diameter of 3 − 5 μm are commonly used. At the beginning of the gradient elution, a mobile phase with a low eluting strength, usually composed of a mixture of chloroform and methanol [41,111] or hexane and 2-propanol [93,109] is employed. The proportion of the component with stronger eluting strength (methanol or 2-propanol depending on the solvent system used)

is then increased throughout the elution. Typical mobile phase additives used are ammonium formate, ammonium acetate, acetic acid, triethylamine or ammonia [41,33,93,109–111]. The volume of water in the mobile phase is usually between 0.5 to 3 % *v/v* [33]. To be precise, such a chromatographic system is termed aqueous NPLC (ANPLC). For example, the ANPLC system was used to the characterise rat brain PLs using a silica gel column and a mobile phase composed of chloroform, methanol, acetic acid, triethylamine and water in gradient elution that allowed for the separation of eight PL classes including: PGs, CLs, PIs, PEs, PCs, PAs and SMs, as well as PSs, the class characteristic for brain tissues [41].

 In recent years, HILIC has become an increasingly popular technique for the analysis of PL composition. The most commonly used columns are 100 – 150 mm long with a diameter of 2 – 4.6 mm, packed with either unmodified silica gel [99,106] or modified with diol [112] or amine groups [111]. The mobile phase is usually a mixture of acetonitrile and water [68,106,112]. During the analysis, the water content increases usually up to 40 % *v/v* ensuring a stronger elution strength in the HILIC system [113,114]. The appropriate pH of the mobile phase is obtained by adding 0.1 – 0.2 % *v/v* formic acid, 5 – 10 mM ammonium acetate or 20 mM ammonium formate [33].

In the case of HILIC, the typical analysis time ranges from 15 min for isocratic analyses [115] up to 60 min in gradient elution, where even the separation of positional isomers of individual PL classes, such as LPEs or LPCs, can be observed (**Figure 6**) [116,117]. Unfortunately, this approach is sometimes insufficient for lipidomic studies. Therefore, there is a constant search for faster lipid separation methods that can be successfully coupled with MS. Modern LC-MS methods are based on the use of shorter columns (e.g., 50 mm) with small particle size (sub-two µm particles) at ultrahigh-pressures (up to 1300 bars), which leads to fast analysis and obtaining narrow chromatographic peaks (even less than a few seconds) [118]. The application of fast LC-MS method in the lipidome study of *Eimeriafalciformis* sporozoites and *Toxoplasma gondii* tachyzoites allowed for separation of PGs, PIs, PEs, inositol phosphorylceramides, ethanolamine phosphorylceramides, PSs, phosphatidylthreonines, PCs and SMs in a runtime of 3.5 min [119]. Another technique enables comprehensive lipidomic analysis within very short time is ultrahigh-performance supercritical fluid chromatography (UHPSFC). This technique coupled with MS has been successfully applied in the study of total

lipid extract of porcine brain and allows detection of close to 30 nonpolar and polar lipid classes, including PLs in a runtime of 6 min [120].

However, attention should be paid to the fact that during one-dimensional separation of PL classes, native and oxidized species belonging to the same class tend to co-elute [41]. Therefore, it seems necessary to re-separate the fractions of individual PL classes by means of another chromatographic system, as shown in the next sections.

5.2.2. Separation of individual PL species

As mentioned above, RPLC supports the separation of individual PL class species according to the length and unsaturation degree of FA chains present in the PL structure as well as in the terms of the type and degree of their oxidative modification [46,106,107]. For this purpose, standard chromatographic columns with a length of 150 – 250 mm packed with silica gel modified with C18 groups, less frequently C8 and C30, are used. The mobile phase is composed of solvents such as acetonitrile, isopropanol, tetrahydrofuran, methanol or water, while the modifiers include ammonium acetate or choline chloride [106,121]. The analysis time in the RPLC technique ranges from 15 min [107] up to 80 min for untargeted analysis for comprehensive characterisation of lipids present in the samples [106]. In the case of PL species analysis by means of LC-MS, fast separation method employing short columns with the length of 50 mm with small particle size (e.g., 1.7 μm) are becoming more and more popular. For example, the application of fast LC-MS/MS method enables comprehensive untargeted lipidomic analysis of oxidized PL species in human embryonic kidney cell line HEK293 as well as primary mouse peritoneal macrophages [86]. This example shows that despite the use of tandem MS, prior separation of lipids is highly recommended for quantitative analysis of oxPLs, which is caused by their large diversity and generally low abundance in biological and food samples [43]. Additionally, this approach allows the identification of oxidized species based on two independent parameters: the *m/z* values and differences in the retention time [25].

The oxidation of unsaturated FAs present in the structures of PLs decreases their hydrophobicity compared to the native counterparts. Consequently, in the RP mode, the retention time of PL molecules depends on the degree of FA oxidation. A higher oxidation degree shortens the elution time of the modified compounds [66,87,108,122]. Separation of oxPLs from their native counterparts can also achieved by means of HILIC [123]. Among the

advantage of such approach, the authors mention better chromatographic separation compared to the RP method developed by them and less ionization suppression effects [123].

5.2.3. Two-dimensional liquid chromatography

As mentioned above, PLs consist a large number of individual compounds that differ in both the structure of the hydrophilic group and the FA composition in the lipophilic fragment. Therefore, their complete separation by means of one-dimensional LC (1D-LC) is practically unachievable. The development of chromatographic techniques over the past decade contributed to an increase of interest in the analysis of PL fraction by two-dimensional LC (2D-LC).

In order to fully understand the application of 2D LC in a comprehensive analysis of PLs, its various types have been briefly characterized. Based on the number of peaks analysed, two different strategies to implement 2D-LC technique are available. In the case of *comprehensive* 2D-LC (LC×LC), all analytes that are eluted from the first dimension ([1]D) column are injected onto the second dimension ([2]D) column. Thus, all peaks eluted from the [1]D column are fully sampled. In the case of *heart-cutting* 2D-LC (LC-LC), analytes eluted as one or few peaks are selected and a fraction of a given peak or peaks is collected and then injected onto the [2]D column [124]. In addition, based on temporal implementation, three types of 2D-LC are available: *online*, *offline* or *stop-and-go*, also known as *stop-flow*[125]. During *online* analysis, the eluate from the [1]D column is collected in the loop and after filling it, the fraction is immediately injected onto the [2]D column. In this case, the [2]D analysis time is limited to the collection time of the fractions from the [1]D column. Therefore, this approach is confined to very short chromatographic columns (e.g., 10 mm) during the [2]D separation [126]. In the *offline* approach of 2D-LC technique, [1]D column eluates are collected manually or with fraction collectors and the fractions are stored. When needed, the mobile phase from the [1]D fractions can be evaporated, and the analytes can be redissolved in the solvent compatible with the [2]D system [106,117]. Additionally, this approach allows to concentrate samples. Comparing both schemes, the *online* approach is clearly faster and can be fully automated, while the *offline* system enables a higher resolving power as a result of using longer columns during [2]D separation [125]. In the case of the *stop-flow* mode, the [1]D and [2]D analysis are carried out rotationally. During [1]D separation, the eluate is collected in the loop [107]. After loop filling, the [1]D pump is interrupted, while the eluate collected in the loop is reinjected onto [2]D column. Once [2]D separation is completed, the

[1]D pump is restarted and the [1]D separation is continued until the loop is filled again. The cycles are repeated as often times as required [107,125].

Due to the high resolving power of 2D-LC, it is successfully used in the field of lipid science. For instance, the application of *online* comprehensive normal phase × reverse phase 2D LC (NP×RP 2D-LC) method enabled the identification of 721 lipid compounds in a sample isolated from rat peritoneal surface layer, among which numerous PLs differing in the structure of hydrophilic group as well as FA composition were distinguished [126]. The *offline* approach of 2D-LC technique is also commonly used in the analysis of lipids, especially PLs. The advantage is that longer chromatographic columns used during [2]D ensure higher resolution of, assuming the use of the same particle size of stationary phase and eluent flow rate. Therefore, the *offline* system can be successfully employed during untargeted analyses for comprehensive characterisation of lipid fractions in food and biological samples. The application of *offline* HILIC-RP 2D-LC system in the analysis of hen egg yolk, soybeans or porcine brain tissue allowed for identification of over 150 different lipid compounds. Among them, numerous PLs have been detected, including various PL classes (PGs, PIs, CLs, PEs, PCs and SMs), their lyso forms (LPGs, LPIs, LPEs and LPCs) as well as PE and PC plasmalogen forms possessing vinyl ether fatty alcohols at the *sn*-1 position of the glycerol backbone [106].

An important advantage of the LC×LC techniques is the separation of native PLs from their oxidized derivatives within individual PL classes [41]. This goal was accomplished by means of [1]D separation of PLs using NP mode and collection of fractions corresponding to individual PL classes. Then, the mobile phase was evaporated under a stream of nitrogen, the analytes were dissolved in the appropriate solvent and individual PL classes were subsequently analyzed in the second dimension using RP mode [41].

The third mentioned 2D-LC approach that can be implemented for the PL analysis is the *stop-flow* system. An example of the application of *stop-flow* comprehensive 2D-LC method is the analysis of PLs present in cow's milk and plasma samples. For this purpose, a loop holding a volume of 100 µL, which provided one-minute collection of eluate from the [1]D HILIC column was employed. After filling the loop, the collected fraction was injected directly onto the RP column. The [2]D analysis ensured the separation of molecular species differing in the length and degree of unsaturation of FAs present in diacyl moieties. The application of the *stop-and-go* HILIC×RP 2D-LC method enabled separation of 6 PL classes (PIs, PEs, PSs, PCs, SMs

and LPCs) in the first dimension and determination of up to 16 individual species differing in FA composition (**Figure 7**) [107]. Nevertheless, one of the disadvantages of *stop-and-go* implementation is the extended time of the analysis, which can last up to 6 hours [127]. In addition, the analytes that are present for such a long time in the ^{1}D column may diffuse even though the mobile phase flow is stopped. Consequently, this leads to weakening of the ^{1}D resolving power [125].

6. Detection of PL

6.1. Basic detectors coupled with LC used in PL analysis

In the case of native PL analysis, spectrophotometric detectors are practically unusable due to the lack of groups in their structures with characteristic wavelength absorption. Therefore, absorption in the $190-210$ nm range results from the presence of double bonds as well as some functional groups, such as carboxyl, phosphate, and amine [128] is occasionally used [100]. However, the extinction coefficient is strongly dependent on the unsaturation degree of FAs within PL structure, which makes the quantitative analysis practically impossible [129].

In contrast to native molecules, the spectrophotometric detectors can be successfully applied for the analysis of oxPLs. The homolytic hydrogen atom abstraction from the methylene group of PUFAs that occurs during oxidation as a result of electron rearrangement leads to the formation of conjugated dienes [9]. This structure exhibits the characteristic absorption at the wavelength around 230 nm. Therefore, LC coupled with spectrophotometric detector set at 232-234 nm is used for the detection of numerous primary (hydroperoxides) and secondary (epoxides, alcohols and ketones) products of PL oxidation [130–132]. We applied this approach during RP analysis of PE and PC fractions obtained by HILIC fractionation of enzymatically oxidized PLs isolated from hen egg yolk, as shown in the **Figure 8d-e**[99]. However, it should be noted that application of LC-UV-MS approach, where low sample concentrations are exposed to high UV doses in a detector cell may have served impact on the structure of analytes [133]. Such alterations can be particularly probable in the case of analysis of unstable lipid oxidation products such as oxPLs and consequently lead to generation of artifact signals in the MS spectrum.

Fluorescence detectors are another type of detectors sometimes coupled with LC during PL analysis. In this case, a pre-column or post-column derivatization of analytes is necessary.A number ofnon-specific and specific derivatizing reagentscan beemployed.For example, 1-

dimethylaminonaphthalene-5-sulfonyl chloride or succinimidyl 2-naphthoxyacetate are used for pre-column derivatization of PL molecules with primary amino group, such as PEs and PSs.In the case ofPAs, an example of a pre-column derivatizing reagent is 3-(9-anthroyl) diazo-2-propene [134].For post-column derivatization, the most common are non-specific probes that form non-covalent complexes with the hydrophobic fragment of PL molecules, but also with other lipid compounds such as TAGs. The formation of the complex leads to a rapid increase of probe fluorescence,upon the change of the environmentfrom hydrophilic to hydrophobic. An example of such non-specific post-column probes are 1,6-diphenyl-1,3,5-hexatriene (DPH) or 2,5-bis(5-*tert*-butyl-benzoxazol-2-yl)thiophene (BBOT)[121,135].

 The type of detectors widely used for the qualitative and quantitative analysis of lipids, including PLs, are aerosol detectors such as evaporative light scattering detector (ELSD) and charged aerosol detector (CAD). In this type of detectors, the eluate is nebulized in an inert gas stream resulting in the formation of a primary aerosol, which then flows into the drying tube, where the mobile phase is evaporated off [136]. In the case of ELSD, the resulting secondary aerosol is transferred into the detection chamber, where the beam of light crosses the cloud of analyte and the scattering of light is measured by a photomultiplier. During analysis using CAD, the dried particles collide with a stream of positively charged gas (e.g., nitrogen), which forces the transfer of the charge onto the analyte molecules. Finally, the particles are transported via ion trap to the collector, where their charge is measured by an electrometer. The most known disadvantage of ELSD is the narrow range of analyte concentration for which the linear response of the detector is achieved. A similar disadvantage is observed also for CAD, yet the linear response range is still approximately 10 times broader [136]. Additionally, ELSD response is also depended on ionic strength and thus its application may cause some difficulties during gradient elution program.

Despite these facts, aerosol detectors are widely used, especially in the field of food science and food industry, where in-depth PL analysis using advanced technologies is not always necessary. Both ELSD and CAD have been successfully applied in the analysis of food samples such as milk [68,85] or egg yolk (**Figure 8a**) [99,137]. In our experiments, we applied CAD to profiling of oxPE and oxPC species formed during lipoxidase-catalysed peroxidation of egg yolk PL fraction (**Figure 8b-c**) [99]. During separation of the fractions of individual PL classes in RP

mode, CAD enabled monitoring of both oxidized and non-oxidized PLs, which is not possible using DAD (**Figure 8d-e**).

6.2. Mass spectrometry

In recent years, shotgun MS, TLC-MS and LC-MS have become the key analytical techniques used in the field of lipid science. The application of low-energy ionization techniques, such as ESI and MALDI, has revolutionized the use of MS in the analysis of labile biomolecules including PLs and especially product of their oxidation [43]. Consequently, these techniques paved the way to the development of modern lipidomics and oxidative lipidomics [46]. However, the analysis of oxPLs is still a big challenge and so far there is a lack of universal methods to analyse them in a wide range of molecular masses and different concentration levels [25].

The shotgun MS, in which ESI is generally employed [138], is mainly used in targeted analysis aimed at detecting a set of selected compounds. However, direct injection of a complex mixture of compounds may reduce the ionization efficiency, especially those that are present in low concentration such as oxPLs [139]. Therefore, as mentioned earlier, this approach is not recommended in the analysis of PL oxidation products [43] or at least the use of high-resolution MS (the details of the technique are given in Section 6.2) is indicated [25]. Direct injection of complex sample into low-resolution instruments can limits the isolation of low-abundant ions (e.g., oxPLs ions), which are necessary for tandem mass spectrometry experiments [25].

There are also some limitation in the application of MALDI technique in the analysis of PLs and particularly oxPLs in complex samples, which is due to the fact that this technique is not compatible with online separation techniques needed to minimalize analyte suppression effects and to increase the dynamic range of detection [43,64]. Although some reports indicate the application of this technique in native and oxidized PL studies, but these reports often concern model substances rather than complex samples. For example, the MALDI-TOF MS method was used to determine the effect of model PL oxidation on the kinetics of digestion catalysed by phospholipase A²[140]. A helpful solution in the case complex samples can be separation of lipids of interest (e.g., oxidized species) already at the sample preparation stage followed by their analysis using MALDI. An example of such sample preparation technique is μSPE, as described earlier [64].

Another approach in the MS analysis of PLs involves their initial separation by means of HPTLC technique followed by direct PLs extraction from appropriate chromatogram zones of the plates and their injection into mass spectrometer. This can be achieved by application of TLC-MS Interface, e.g., delivered by CAMAG, Switzerland, as shown in the study of hypochlorous acid-induced oxidation products of PC(C16:0/18:1) [141]. Alternatively, PLs separated by HPTLC technique can be directly loaded from the plate into the mass spectrometer using ionization techniques such as MALDI or desorption electrospray ionization (DESI). For example, the application of TLC coupled with MALDI-TOF MS device enabled comprehensive characterization of PLs isolated from hen egg yolk [142] and porcine brain [143]. The detection of individual PL classes was possible even for analytes occurring at levels less than 1 % of the total PL fraction (e.g., PIs in hen egg yolk), which corresponded to the detection limit of about 400 pmol [142]. Moreover, previous studies employing normal phase TLC technique coupled with MALDI-TOF MS revealed that FA composition within individual PL classes affected their position within the band corresponding to a respective class. The PE and PC species containing shorter and more unsaturated acyl residues were characterized by lower retention factor (*Rf*) values [144]. In turn, LC coupled with MS detection is usually associated with employment of ESI; less common is atmospheric pressure chemical ionization (APCI).

In the analysis of native and oxidized PLs, low-energy ionization techniques such as MALDI or ESI are commonly used. These techniques do not allow imparting enough quantities of energy onto the subject molecule to induce fragmentation in the ion source in opposition to hard ionization techniques, where in-source ion fragmentation takes place introducing complexity to the MS spectrum and difficulty to molecule identification. Considering the above, acute identification of PL species, which despite the different FA composition and various oxidative modifications have exactly the same molecular mass, is difficult to achieve even using high-resolution MS. For example, the structural isomers of PC(C16:0/20:4) with one hydroperoxide group or two hydroxy groups are characterized by the same molecular mass (*m/z* 814.6, [M+H]$^+$). For this reason, tandem mass spectrometry (MS/MS), where controlled ion fragmentation is achieved e.g., by collision-induced dissociation (CID), is a powerful tool in the analysis of native and oxidized PLs in complex samples. The MS/MS technique ensures the fragmentation of ions selected in the first mass analyzer as a result of collision with an inert gas in the collision cell. In the next step, fragmented ions are analyzed in the second mass analyzer

[145]. On the one hand, interpretation of MS/MS spectra is a big challenge, but on the other hand this approach provides unique information on the structure of molecules particularly useful in the identification of structural and positional isomers. In the case of oxidized species, correct interpretation of MS/MS spectrum can contribute to the in-depth structural characterization including position of oxidized acyl chain in glycerol moiety, the side of modification along the unsaturated acyl chain as well as the nature of oxidation group(s) [146,147]. Moreover, some authors proposed the application of ozone-induced dissociation coupled with MS/MS to examine the of double bound localization in positional isomers. For example, Deeley et al. used this technique to distinguish PL plasmalogens, which allowed to find the following 1-O-alkyl PE and PS species in human lens: pPE(16:0/9Z-18:1), pPE(11Z-18:1/9Z-18:1), and pPE(18:0/9Z-18:1), pPS(16:0/9Z-18:1), pPE(11Z-18:1/9Z-18:1), pPE(18:0/9Z-18:1) [148].

As a result of parent ions fragmentation and analysis of generated fragment ions, it is possible to identify FAs in the *sn*-1 and *sn*-2 positions of individual PL species [42,149]. For example, shotgun MS/MS method was employed to identify the long-chain oxidation products of PCs that differed in length and unsaturation degree of FA present in the *sn*-2 position of glycerol [150]. In the case of complex biological samples, high-resolution MS/MS analysis of PLs and especially oxidize species is preceded by separation by means of fast LC. For example, application of LC-MS/MS method enabled both qualitative and quantitative analysis of oxidized species of individual PL classes in mouse primary macrophages. The total amounts of oxPCs, oxPEs and oxPIs per one million of cells were 1180.3, 936.5 and 80.7 fmol, respectively [86]. The MS/MS technique has also been used in the analysis of short-chain oxPLs formed as a result of cleavage of the unsaturated FA including carbonyl products, such as saturated and unsaturated aldehydes, hydroxy-aldehydes, carboxylic acids, and hydroxy carboxylic acids esterified to glycerol backbone [146,147,150].

In the field of lipid science, various types of mass analyzers can be employed. Generally, the mass analyzers can be divided into low-resolution quadrupole (Q), linear ion trap (IT) and high-resolution instruments embracing time-of-flight (TOF), quadrupole time-of-flight (Q-TOF) or triple quadrupole (QqQ). The latter are characterized by mass resolving power (determined at full-width half-height maximum) higher than 10,000 [151] and are definitely more popular in lipidomic studies [33]. However, each of them has some advantages and drawbacks concerning qualitative and quantitative analysis of lipids. For example, QqQ are

commonly used for quantification of native and oxidize PLs. In turn, for the structural characterization of unknown lipids, high-resolution MS such as TOF based mass analyzers or Fourier transform ion cyclotron resonance (FTICR) are widely employed [43]. Moreover, modern high-resolution instruments equipped with fast scan speeds and polarity switching, like some Orbitrap instruments, are highly recommended for detecting of low-abundant oxidized PL species in both positive and negatively ion mode with high throughput [25]. However, high-resolution MS/MS instruments, which do not allow simultaneous scanning in positive and negative ion mode during the analysis, are also successfully used in the analysis of oxPL. For example application of linear ion trap (LTQ) - Orbitrap mass spectrometer equipped with an electrospray ion source enabled the identification of 46 oxidized PC species in oxidized LDL [123].

In addition to untargeted analysis of native and oxidized PLs, targeted strategies are commonly used in lipidomic studies. In the case targeted analysis of choline-containing PL classes, such as PCs and SMs, the most popular approach is precursor ion scanning (PIS) for *m/z* 184 in the positive ion mode [25,152]. This approach takes advantage of the high intensity observed for product ion (fragment) at *m/z* 184 corresponding to protonated ion ([MH]+) of the polar head, in this case choline. For other PL classes, PIS or neutral loss scanning (NLS) acquisition modes can be conducted [153]. Exemplary parameters applied during exploratory analysis of PLs present in human serum for individual classes are as follows: PE, NLS 141 Da (positive ion mode); PS, NLS 185 Da (positive ion mode); PA, NLS 98 Da (positive ion mode); PG, NLS 172 Da (positive ion mode); and PI, PIS *m/z* 241 Da (negative ion mode) (**Figure 9**) [152] In turn, multi-reaction monitoring (MRM) experiments are commonly used e.g., for screening known oxPLs [154]. The advantage of targeted approach is higher resolution and better sensitivity [152], however limited range of native and oxidised PL species screened in each acquisition cycle is the main drawback. Therefore, during multiple PL classes analysis, several injection can be necessary, which in consequence can require a larger sample volume [25].

6.3. Ion mobility spectrometry

The differentiation of PL isomers is often difficult even using ESI-MS/MS technique. For this reason, another technique – ion mobility spectrometry (IMS) – is more useful in some applications. So far, this technique has been successfully used in the analysis of PL species

of individual classes differ in the structures of acyl chains, but it has been not applied to the study of oxidative modifications along the unsaturated FA chain [155]. In general, similarly to mass spectrometry, the first stage involves ionization of the analytes. Then, the generated ions are introduced into to the analyzer, where they are differentiated based on they mobility within a pressurized chamber under the influence of an electric field. The main difference between IMS and MS is the fact that the measurement of the ion drift time occurs within a pressurized chamber, while the measurement of m/z ratio takes place in the absence of gas. The mobility of ion through the chamber depends mainly on the frequency of the collision between ion and neutral gas. Therefore, the ion mobility of a compounds under ideal experimental conditions depends on its size and shape [156]. In addition, the IMS is usually coupled with mass spectrometry (IMS-MS) and enables full differentiation of ions tacking into account size, shape, mass and charge [33]. Identification of individual compounds is carried out based on m/z ratio and drift time (usually expressed in milliseconds) [157]. The application of ESI-IMS-MS technique in phospholipidomic research enables the differentiation of PL species of individual class that differ in the position of acyl chain present in the structure (regioisomers). Therefore, this approach eliminates the need to use regiospecific enzymes such as phospholipase A[1] and A[2] to determine the position of the FAs in PL structure. Furthermore, the ESI-IMS-MS technique makes it possible to separate PL class species that differ in localization of the double bound in the structure of acyl chain as well as the conformation of double bound (*cis-trans* isomers).

Another technique used in the analysis of PLs is MALDI-IMS-MS, which enables a fast (hundreds of microseconds) analysis of complex mixtures of PLs. The changes in drift times of PLs result from differences in acyl chain length and degree of unsaturation and the nature of the hydrophilic group [158]. In addition, previous studies indicate that formation of alkali PL adducts, formed upon the addition of LiCl, NaCl, KCl or CsCl to the matrix, leads to a significant shift towards lower collision cross sections in comparison with protonated PL species with a similar m/z. Consequently, the shift in the mobility of ions produced by alkali cationization is observed, which is particularly evident in the case of cesium ion. Therefore, the addition of cesium salt to the matrix allows the identification of PL species, which otherwise would not be observed [158]. Exemplary overlay of two-dimensional MALD-IM-MS plots obtained for rat brain PLs with and without cesium ion is shown in **Figure 10**.

7. Data analysis by means of bioinformatic tools

One of the main challenges for lipidomics and foodomics, in particular for MS-based approaches, are computational and bioinformatic requirements for handling large amount of data generated during analysis. A typical workflow in lipidomics begins with sample preparation followed by data acquisition, processing of raw lipid data set and lipid identification using databases such as LIPID MAPS (http://www.lipidmaps.org) [159]. Another important step in lipidomic data analysis is application of appropriate chemometric methods, which allow for selection of key molecules (discriminators) that characterize a specific phenotype. For this purpose, classical statistical tests such as ANOVA methods, principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) are commonly used [160]. Moreover, for complex lipid data sets, generated e.g., during untargeted lipidomics analysis using two-dimensional chromatography coupled with MS, a combination of multiway methods, such as multivariate curve resolution (MCR) can be applied [160]. Recently, the regions of interest multivariate curve resolution (ROIMCR) procedure, which enables compressing and processing LC-MS data, was employed in lipid standards analysis, including PLs. This approach consists of a preliminary selection of the regions of interest of the LC-MS data followed by their throughout chemometric analysis [161]. The bioinformatic analysis includes also the interpretation of observed lipid changes in the biochemical context, which is known as a pathway analysis. An example of database providing lipid metabolic pathway information is Kyoto Encyclopedia of Genes and Genomes (KEGG) (https://www.genome.jp/kegg).

The experimental strategy used to acquire MS data affects data processing and information interpretations. Therefore, different bioinformatic lipid tools can be employed for the analysis of shotgun and LC-MS data [162,163]. Among bioinformatic tools, approaches using reference libraries followed by spectrum similarity searches can be distinguished. Examples of such tools are MassBank (https://massbank.eu/MassBank/), Human Metabolome Database (http://www.hmdb.ca/), which contain both MS and MS/MS spectra of various metabolites, including PLs. In addition, MassBank enablesincorporation of self-created reference spectra [162]. Nevertheless, the lipid identification rate using this databases is usually low, due to the limited amount of empirically generated MS/MS spectra [164]. Therefore, bioinformatic tools based on *in silico* generated tandem MS spectra, such us

LipidBlast (http://fiehnlab.ucdavis.edu/projects/LipidBlast) [164], are employed in lipidomic studies. In this approach, the identification of lipids is obtained by fitting experimental fragment

m/z values with simulated library *m/z* values using *in silico* fragmentation libraries. Another open sources software with *in silico* generated libraries is LipidMatch (http://secim.ufl.edu/ secim-tools/lipidmatch/) [165], which was introduced in 2017 and includes over 500,000 lipid species, across over 60 lipid categories, including PLs. Furthermore, LipidMatch includes a list of potential oxidized FAs (both long- and short-chain) that can be incorporate in the structure of lipids. In turn, the open-source software tool entirely dedicated to the prediction and identification of oxPLs is LPPtiger (https://home.uni-leipzig.de/fedorova/software/lpptiger/) [166], which was proposed in 2017. The LPPtiger workflow uses three unique algorithms: *in silico* oxidation, theoretical CID fragmentation and multi-score-based identification. In this way, the software can predict oxidized lipids in a given lipidome and identify corresponding predicted structures. A powerful bioinformatic tool is also LipidSearch (Thermo Fisher Scientific), which can be used for analysis of large amount of MS data obtained for both shotgun and LC-MS approaches. This software enables automatic identification and relative quantification of cellular lipids [163]. In addition, a number of other bioinformatic tools such as Greazy[167], LipidView (formerly LipidProfiler) [168] or LipidXplorer[169] are used for MS data processing during PL analysis.

Although the use of the bioinformatics tools mentioned above seems to be an invaluable step during lipid analysis, the databases may still be incomplete regarding to the tandem MS spectra of oxPLs, which can limit their identification. In addition, it should also be noted that oxPLs are usually less abundant and consequently may generate MS/MS spectra with lower signal to noise ratio. Therefore, the analytical approaches developed should include specific selection criteria, e.g., isotopic envelope, for more efficient identification of oxPLs even in samples with low ratio of oxidized to native PLs [25].

8. Imaging techniques

8.1. Mass spectrometry imaging

From the biological point of view, apart from the content of individual lipid species, their distribution in a given biological system may be even more important. Therefore, there is a growing interest in imaging techniques such as mass spectrometry imaging (MSI). This label-free technique enables for studying *in situ* localization, structure characterization and relative quantification of multiple lipids in biological samples. The spatial mapping of the distribution of

cellular or tissue lipids is achieved as a result of MS data acquisition from the defined pixels of the sample followed by processing of lipid MS data set [170]. The analysis efficiency depends on several parameters such as the MS instrument configuration, number of shots fired per pixel, the distance between pixels or the data acquisition rate [171]. One of the most commonly used ionization techniques coupled with MSI are MALDI and DESI, which enable direct lipid analysis on the sample surface without time-consuming and laborious extraction steps requires for ESI [155].

The MALDI-MSI technique has proved to be a powerful tool in PL analysis of animal and human tissues such as brain, colon, heart or liver [170] as well cultured cells [172]. Moreover, tracking changes in the cellular and tissue distribution of PLs enabled selection of molecular features most indicative of a given disease state, and consequently can provide insight into disease prediction and monitoring of its progression. An example of this approach was the monitoring of PL changes in the brain of mouse model of Alzheimer's disease associated with development of this disorder. As a result of disease progression, changes in the distribution of various lipid species including PCs, SMs, PIs and LPCs were observed. The lipid remodelling included several regions of the mouse brain such as frontal cortex, hippocampus and subiculum [173]. Another application of MSI technique in lipidomics is to study changes in the PL distribution between normal and cancerous tissues, which may be useful in clinical practise to identify tumor margins after surgical resection. This approach was tested on example of gastric cancer operations in direct comparison with margin assessment by frozen-section histopathology [174]. The statistical analysis indicated that distribution disorder of CL(1'-[18:2/18:2],3'[18:2/18:2]), PE(36:1) and PS(36:1) is diagnostic feature for distinguishing normal gastric tissue from benign and malignant tumor tissues [174]. Differences in the PL distribution were also observed between normal liver tissue and colon cancer liver metastasis, where mainly the increase of SM(16:0) in the case of cancerous tissue was observed [175]. The PL imaging technique also enables discrimination of tumor subtypes and grades. For example, the DESI-MS technique was used for rapid classification of human brain tumors, where differences in PS and PI species were mainly observed [176]. In turn, air flow-assisted ionization (AFAI) MSI technique allowed for molecular distinguishing of breast invasive ductal carcinoma and breast ductal carcinoma *in situ*. Changes in the abundance of four ions corresponding to PL species (*m/z* 706.5, PC(30:0); *m/z* 718.6, PC(32:0) or PE(35:0); *m/z* 724.5, PE(34:1); and

m/z 730.5, PC(32:2)) were observed between tumor subtypes as well as depending on grade of breast cancer (**Figure11**) [177].

Additional exciting application of MSI technique is spatial imaging of oxPLs in tissues, which can contribute to advanced understanding of pathogenesis and progression of numerous diseases. In order to investigate the distribution of oxPLs in the spinal cord tissue from the lumbar region of adult rats, the high-selective MALDI MS^n imaging technique has been used. The application of the mentioned technique allowed for identification and localization of short-chain products of PL oxidation such as PC(18:0/9:0 COOH) and PC(16:0/11:0 COOH) [178]. The MS-based imaging was also used to identify and determine distribution of oxPL in mouse organs such as brain, kidney and liver [179]. Moreover, the effect of different sample storage conditions on formation of oxPLs was determined. The imagining experiments indicated that global formation of oxidized species, such as PC(16:0/9:0 COOH), PC(16:0/11:0 CHO) and PC(16:0/11:0 COOH), across the tissue was observed within 24 h under ambient conditions. It was also shown that PL oxidation is inhibited if the samples are stored at -80 °C under nitrogen atmosphere [179].

8.2. Confocal imaging technique

The analytical techniques described above can be coupled to the investigation of the lipid maps in cells. In many cases, changes in the composition of PLs in cells due to nutritional overload or oxidative stress translate into biophysical changes in cellular membranes. Recently, it was shown that the fluorescent probe Laurdan can be used in confocal imaging to follow alterations in the fluidity of the plasma membrane and insulin granule membranes in insulin secreting beta cells exposed to high glucose and/or high palmitic acid concentrations (**Figure 12**) [180]. Laurdan, which incorporates into the lipid phase in membranes, changes emission intensity in a way correlating with the biophysical state of its environment. Its excited-state relaxation is independent of the head-group type in PLs, but is sensitive to the degree of saturation of FA moieties present in PLs and freedom of mobility of water molecules in the membrane bilayer. It was shown before that high glucose and/or high palmitic acid incubations activate phospholipase A_2 and induced PLs remodelling in beta cells by exchanging polyunsaturated FA and introducing saturated FA and monounsaturated FA [181,182], which led to significant changes, predominantly in the fluidity of the membrane of insulin granules.

Also the real time quantitative analysis of cellular lipid maps by confocal spectral imaging of intracellular micropolarity was performed [183]. For this purpose, the solvatochromic and lipophilic probe Nile Red, which shifts its emission spectrum from yellow to red when the degree of polarity of the lipid environment increases, was used. The adapted spectral phasor analysis helped to determine pixel-by-pixel the degree of lipid polarity, and to mapp insulin secreting beta cells to provide accurate representations of the proportion of non-polar, polar and highly polar lipids in the cells and created a topographical image of the cellular distribution of the various classes of lipids in the plasma membrane, insulin granules and lipid droplets in the cells.

Fluorescence probes have also been used in the study of membrane lipid oxidation using fluorescence and confocal microscopy. An example of such probe is C11-BODIPY[581/591], which is a FA analogue with emission maximum in the red range of visible spectrum. The previous research using living rat-1fibroblasts showed that the fluorescent probe is distributed heterogeneously throughout cellular membranes, with predominant localization in the perinuclear region of cell. The probe was not specific for any particular organelle, such as mitochondria, peroxisomes, lysosomes and Golgi apparatus, while lower probe incorporation was observed for rat-1fibroblast plasma membrane [184]. As a result of free radical-induced oxidation of butadienyl moiety of the probe, the fluorescence emission maximum shifts from 595 nm (red fluorescence) to 520 nm (green fluorescence) [184]. Therefore, the C11-BODIPY[581/591] is widely used in the study of lipid oxidation of model membrane systems as well as cellular membrane lipid damage, e.g., caused by oxidative stress. The application of mentioned fluorescent probe revealed that the membrane oxidation of lymphoma cells leads to increase of human secreted phospholipase A^2 activity, which contribute to the explanation of mechanism of thapsigargin induced cell apoptosis [185]. The previous research indicated also that C11-BODIPY[581/591] was exclusively sensitive to free radical species formed as a result of hydroperoxides breaking-down, but not to hydroperoxides *per se*[184]. In contrast to C11-BODIPY[581/591], Liperfluo responds only to membrane lipid hydroperoxides. This probe reduces (phospho)lipid hydroperoxides to their hydroxy-homologues, which results in the formation of highly fluorescence product – LiperfluoOx – with emission maximum at 535 nm [186]. Similar to C11-BODIPY[581/591] fluorescence probe, Liperfluo can be used to living cell imaging. An example of such approach is the use of Liperfluo in the study of cell ferroptosis.

The results obtained with redox lipidomics, reverse genetics, bioinformatics and systems biology showned that only PEs present in the endoplasmic reticulum associated compartments underwent oxidation with the specificity toward two fatty acyls: arachidonic: arachidonoyl and adrenoyl [187].

The use of such probes in confocal imaging is complementary to the lipidomic analysis of the various classes of lipids in cells, and in particular PLs, and can synergistically enhance lipid research from cellular to subcellular levels.

Conclusion

The development of advanced analytical techniques, which has taken place in recent years, has significantly contributed to a better understanding of the biological role of PLs in the functioning of cells, tissues, organs and whole organism. This has materialized due to a comprehensive analysis of the endogenous PL species in biological samples that aim at their identification as well as monitoring of their concentration, oxidative modification and cellular distribution. The important aspect is the characterisation of exogenous PLs (including oxidative derivatives) delivered with food, which may affect the functioning of the human body. From the biological point of view, the composition of PL classes, the abundance and position of FAs present in PL molecules, the position and conformation of unsaturated carbon atoms within them as well as oxidative modifications may alter cell functions due to structural and modified signaling pathways. Fortunately, the constantly developing analytical techniques enable the identification and quantitation of numerous PL molecules differing in the structure in ever-shorter time. Nevertheless, it should be noted that the advanced approaches are often preceded by the application of standard techniques for initial characterization of lipid fraction in tested samples, especially in the case of food industry, where the access to advanced equipment is not always warranted.

Conflict of interest

The authors declare that they have no conflict of interest.

References

[1] Küllenberg de Gaudry D, Taylor L a, Schneider M, et al. Health effects of dietary phospholipids. Lipids Health Dis 2012;11:1–16.

[2] Molendi-Coste O, Legry V, Leclercq IA. Why and how meet n-3 PUFA dietary recommendations? Gastroenterol Res Pract 2011;2011:1–11.

[3] Blesso CN. Egg phospholipids and cardiovascular health. Nutrients 2015;7:2731–2747.

[4] Ahn SH, Lim SJ, Ryu YM, et al. Absorption rate of krill oil and fish oil in blood and brain of rats. Lipids Health Dis 2018;17:1–8.

[5] Choi JY, Jang JS, Son DJ, et al. Antarctic krill oil diet protects against lipopolysaccharide-induced oxidative stress , neuroinflammation and cognitive impairment. Int J Mol Sci 2017;18:1–15.

[6] Ursoniu S, Sahebkar A, Serban M, et al. Lipid-modifying effects of krill oil in humans: systematic review and meta-analysis of randomized controlled trials. Nutr Rev 2017;75:361–373.

[7] Burri L, Hoem N, Banni S, et al. Marine Omega-3 phospholipids: Metabolism and biological activities. Int J Mol Sci 2012;13:15401–15419.

[8] Taylor LA, Pletschen L, Arends J, et al. Marine phospholipids - A promising new dietary approach to tumor-associated weight loss. Support Care Cancer 2010;18:159–170.

[9] Wąsowicz E, Gramza A, Hęś M, et al. Oxidation of lipids in foods. Polish J Food Nutr Sci 2004;13:87–100.

[10] Miyamoto S, Dupas C, Murota K, et al. Phospholipid hydroperoxides are detoxified by phospholipase A2 and GSH peroxidase in rat gastric gucosa. Lipids 2003;38:641–649.

[11] Ursini F, Zamburlini A, Cazzolato G, et al. Postprandial plasma lipid hydroperoxides: A possible link between diet and atherosclerosis. Free Radic Biol Med 1998;25:250–252.

[12] Cohn JS, Kamili A, Wat E, et al. Dietary phospholipids and intestinal cholesterol absorption. Nutrients 2010;2:116–127.

[13] Fruhwirth GO, Loidl A, Hermetter A. Oxidized phospholipids: From molecular properties to disease. Biochim Biophys Acta - Mol Basis Dis 2007;1772:718–736.

[14] Ashraf MZ, Kar NS, Podrez EA. Oxidized phospholipids: Biomarker for cardiovascular diseases. Int J Biochem Cell Biol 2009;41:1241–1244.

[15] Ravandi A, Babaei S, Leung R, et al. Phospholipids and oxophospholipids in atherosclerotic plaques at different stages of plaque development. Lipids 2004;39:.

[16] Hammad LA, Wu G, Saleh MM, et al. Elevated levels of hydroxylated phosphocholine lipids in the blood serum of breast cancer patients. Rapid Commun Mass Spectrom 2009;23:863–876.

[17] Kinoshita M, Oikawa S, Hayasaka K, et al. Age-related increases in plasma phosphatidylcholine hydroperoxide concentrations in control subjects and patients with hyperlipidemia. Clin Chem 2000;46:822–828.

[18] Gwak YS, Kang J, Leem JW, et al. Oxidized phosphatidylcholine is a marker for neuroinflammation in multiple sclerosis brain. J Neurosci Res 2007;85:2352–2359.

[19] Bochkov VN, Oskolkova O V, Birukov KG, et al. Generation and biological activities of oxidized phospholipids. Antioxid Redox Signal 2010;12:1009–1059.

[20] Adachi J, Matsushita S, Yoshioka N, et al. Plasma phosphatidylcholine hydroperoxide as a new marker of oxidative stress in alcoholic patients. J Lipid Res 2004;45:967–971.

[21] Hyotylainen T, Bondia-Pons I, Oresic M. Lipidomics in nutrition and food research. Mol Nutr Food Res 2013;57:1306–1318.

[22] Ferreri C, Chatgilialoglu C. Membrane lipidomics for personalized health. Chichester: John Wiley & Sons; 2015.

[23] Ferreri C, Chatgilialoglu C. Role of fatty acid-based functional lipidomics in the development of molecular diagnostic tools. Expert Rev Mol Diagn 2012;12:767–780.

[24] Nicolson GL, Ash ME. Lipid Replacement Therapy: A natural medicine approach to replacing damaged lipids in cellular membranes and organelles and restoring function. Biochim Biophys Acta - Biomembr 2014;1838:1657–1679.

[25] Reis A. Oxidative Phospholipidomics in health and disease: achievements, challenges and hopes. Free Radic Biol Med 2017;11:25–37.

[26] Cifuentes A. Food analysis and foodomics. J Chromatogr A 2009;1216:7109.

[27] Cifuentes A. Food Analysis: Present, future , and foodomics. ISRN Anal Chem 2012;2012:.

[28] Herrero M, Simó C, García-Cañas V, et al. Foodomics: MS-based strategies in modern food science and nutrition. Mass Spectrom Rev 2012;31:49–69.

[29] Gorrochategui E, Jaumot J, Lacorte S, et al. Data analysis strategies for targeted and untargeted LC-MS metabolomic studies: overview and workflow. Trends Anal Chem 2016;82:425–442.

[30] Han X, Gross RW. Electrospray ionization mass spectroscopic analysis of human erythrocyte plasma membrane phospholipids. Proc Natl Acad Sci 1994;91:10635–10639.

[31] Marto JA, White FM, Seldomrldge S, et al. Structural characterization of phospholipids by matrix-assisted laser desorption/ionization Fourier transform ion cyclotron resonance mass spectrometry. Anal Chem 1995;67:3979–3984.

[32] Schiller J, Arnold K. Application of high resolution 31P NMR spectroscopy to the characterization of the phospholipid composition of tissues and body fluids - a methodological review. Med Sci Monit 2002;8:MT205-T222.

[33] Cajka T, Fiehn O. Comprehensive analysis of lipids in biological systems by liquid chromatography - mass spectrometry. Trends Anal Chem 2014;61:192–206.

[34] Ferreri C, Masi A, Sansone A, et al. Fatty acids in membranes as homeostatic, metabolic and nutritional biomarkers: Recent advancements in analytics and diagnostics. Diagnostics 2017;7:1–14.

[35] Fahy E, Subramaniam S, Brown HA, et al. A comprehensive classification system for lipids. J Lipid Res 2005;46:839–862.

[36] Guo Z, Vikbjerg AF, Xu X. Enzymatic modification of phospholipids for functional applications and human nutrition. Biotechnol Adv 2005;23:203–259.

[37] Christie W. Ether lipids. AOCS Lipid Libr 2016;vii-151.

[38] Christie W. Long-chain or sphingoid bases. AOCS Lipid Libr 2016;1–9.

[39] Mukhamedova KS, Glushenkova AI. Natural phosphonolipids. Chem Nat Compd 2000;36:329–341.

[40] Van Nieuwenhuyzen W, Tomás MC. Update on vegetable lecithin and phospholipid technologies. Eur J Lipid Sci Technol 2008;110:472–486.

[41] Samhan-Arias AK, Ji J, Demidova OM, et al. Oxidized phospholipids as biomarkers of tissue and cell damage with a focus on cardiolipin. Biochim Biophys Acta - Biomembr 2012;1818:2413–2423.

[42] O'Donnell VB. Mass spectrometry analysis of oxidized phosphatidylcholine and phosphatidylethanolamine. Biochim Biophys Acta - Mol Cell Biol Lipids 2011;1811:818–826.

[43] Bochkov V, Gesslbauer B, Mauerhofer C, et al. Pleiotropic effects of oxidized phospholipids. Free Radic Biol Med 2017;111:6–24.

[44] Reis A, Spickett CM. Chemistry of phospholipid oxidation. Biochim Biophys Acta - Biomembr 2012;1818:2374–2387.

[45] Zhou L, Zhao M, Bindler F, et al. Identification of oxidation compounds of 1-stearoyl-2-linoleoyl- sn -glycero-3-phosphoethanolamine during thermal oxidation. J Agric Food Chem 2015;63:9615–9620.

[46] Peterson BL, Cummings BS. A review of chromatographic methods for the assessment of phospholipids in biological samples. Biomed Chromatohraphy 2006;243:227–243.

[47] Cruz M, Wang M, Frisch-Daiello J, et al. Improved butanol-methanol (BUME) method by replacing acetic acid for lipid extraction of biological samples. Lipids 2016;51:887–896.

[48] Birjandi AP, Bojko B, Ning Z, et al. High throughput solid phase microextraction: A new alternative for analysis of cellular lipidome? J Chromatogr B 2017;1043:12–19.

[49] Garwolińska D, Hewelt-Belka W, Namieśnik J, et al. Rapid characterization of the human breast milk lipidome using a solid-phase microextraction and liquid chromatography-mass spectrometry-based approach. J Proteome Res 2017;16:3200–3208.

[50] Ulmer CZ, Jones CM, Yost RA, et al. Optimization of Folch, Bligh-Dyer, and Matyash sample-to-extraction solvent ratios for human plasma-based lipidomics studies. Anal Chim Acta 2018;1037:351–357.

[51] Reis A, Rudnitskaya A, Blackburn GJ, et al. A comparison of five lipid extraction solvent systems for lipidomic studies of human LDL. J Lipid Res 2013;54:1812–1824.

[52] Pellegrino RM, Di Veroli A, Valeri A, et al. LC/MS lipid profiling from human serum: a new method for global lipid extraction. Anal Bioanal Chem 2014;406:7937–7948.

[53] Matyash V, Liebisch G, Kurzchalia T V, et al. Lipid extraction by methyl-tert-butyl ether for high-throughput lipidomics. J Lipid Res 2008;49:1137–1146.

[54] Patterson RE, Ducrocq AJ, McDougall DJ, et al. Comparison of blood plasma sample preparation methods for combined LC-MS lipidomics and metabolomics. J Chromatogr B 2016;1002:260–266.

[55] Gil A, Zhang W, Wolters JC, et al. One- vs two-phase extraction: re-evaluation of sample preparation procedures for untargeted lipidomics in plasma samples. Anal Bioanal Chem 2018;410:5859–5870.

[56] Löfgren L, Ståhlman M, Forsberg G-B, et al. The BUME method: a novel automated chloroform-free 96-well total lipid extraction method for blood plasma. J Lipid Res 2012;53:1690–1700.

[57] Löfgren L, Forsberg G, Ståhlman M. The BUME method: a new rapid and simple chloroform-free method for total lipid extraction of animal tissue. Sci Rep 2016;6:27688.

[58] Barrilero R, Gil M, Amigó N, et al. LipSpin: a new bioinformatics tool for quantitative 1H-NMR lipid profiling. Anal Chem 2018;90:2031–2040.

[59] Pizarro C, Arenzana-Rámila I, Pérez-del-Notario N, et al. Plasma lipidomic profiling method based on ultrasound extraction and liquid chromatography mass spectrometry. Anal Chem 2013;85:12085–12092.

[60] Teo CC, Chong WKP, Tan E, et al. Advances in sample preparation and analytical techniques for lipidomics study of clinical samples. Trends Anal Chem 2015;66:1–18.

[61] Khoomrung S, Chumnanpuen P, Jansa-Ard S, et al. Rapid quantification of yeast lipid using microwave-assisted total lipid extraction and HPLC-CAD. Anal Chem 2013;85:4912–4919.

[62] Sarafia MH, Gaudin M, Lewis MR, et al. Objective set of criteria for optimization of sample preparation procedures for ultra-high throughput untargeted blood plasma lipid profiling by ultra performance liquid chromatography−mass spectrometry. Anal Chem 2014;86:5766−5774.

[63] Stübiger G, Aldover-Macasaet E, Bicker W, et al. Targeted profiling of atherogenic phospholipids in human plasma and lipoproteins of hyperlipidemic patients using MALDI-QIT-TOF-MS/MS. Atherosclerosis 2012;224:177–186.

[64] Stübiger G, Belgacem O, Rehulka P, et al. Analysis of oxidized phospholipids by MALDI mass spectrometry using 6-Aza-2-thiothymine together with matrix additives and disposable target. Anal Chem 2010;82:5502–5510.

[65] Liakh I, Pakiet A, Sledzinski T, et al. Modern methods of sample preparation for the analysis of oxylipins in biological samples. Molecules 2019;24:1639.

[66] Spickett CM, Reis A, Pitt AR. Identification of oxidized phospholipids by electrospray ionization mass spectrometry and LC-MS using a QQLIT instrument. Free Radic Biol Med 2011;51:2133–2149.

[67] Ulmer CZ, Patterson RE, Koelmel JP, et al. A robust lipidomics workflow for mammalian cells, plasma, and tissue using liquid-chromatography high- resolution tandem mass spectrometry. In: Lipidomics: Methods and Protocols, Methods in Molecular Biology. 2017. p. 91–106.

[68] Donato P, Cacciola F, Cichello F, et al. Determination of phospholipids in milk samples by means of hydrophilic interaction liquid chromatography coupled to evaporative light scattering and mass spectrometry detection. J Chromatogr A 2011;1218:6476–6482.

[69] Bruun-Jensen L, Colarow L, Skibsted LH. Detection and quantification of phospholipid hydroperoxides in turkey meat extracts by planar chromatography. J Planar Chromatogr 1995;8:475–479.

[70] Hammad SM, Pierce JS, Soodavar F, et al. Blood sphingolipidomics in healthy humans: impact of sample collection methodology. J Lipid Res 2010;51:3074–3087.

[71] Gonzalez-Covarrubias V, Dane A, Hankemeier T, et al. The influence of citrate, EDTA, and heparin anticoagulants to human plasma LC–MS lipidomic profiling. Metabolomics 2013;9:337–348.

[72] Chua EC, Shui G, Lee IT, et al. Extensive diversity in circadian regulation of plasma lipids and evidence for different circadian metabolic phenotypes in humans. Proc Natl Acad Sci 2013;110:14468–14473.

[73] Kasukawa T, Sugimoto M, Hida A, et al. Human blood metabolite timetable indicates internal body time. Proc Natl Acad Sci 2012;109:15036–15041.

[74] Aviram R, Manella G, Kopelman N, et al. Lipidomics analyses reveal temporal and spatial lipid organization and uncover daily oscillations in intracellular organelles resource lipidomics analyses reveal temporal and spatial lipid organization and uncover daily oscillations in intracellular organ. Mol Cell 2016;62:636–648.

[75] Amézaga J, Arranz S, Urruticoechea A, et al. Altered red blood cell membrane fatty acid profile in cancer patients. Nutrients 2018;10:1–13.

[76] Folch J, Less M, Sloane Strnley GH. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem 1957;226:497–509.

[77] Bligh EG, J. DW. A rapid method of total lipid extraction and purification. Can J Biochem Physiol 1959;37:911–917.

[78] Iverson SJ, Lang SL, Cooper MH. Comparison of the Bligh and Dyer and Folch methods for total lipid determination in a broad range of marine tissue. Lipids 2001;36:1283–1287.

[79] Cequier-Sánchez E, Rodríguez C, Ravelo ÁG, et al. Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures. J Agric Food Chem 2008;56:4297–4303.

[80] Matyash V, Liebisch G, Kurzchalia T V, et al. Lipid extraction by methyl- tert -butyl ether for high-throughput lipidomics. J Lipid Res 2008;49:1137–1146.

[81] Mawatari S, Hazeyama S, Morisaki T, et al. Enzymatic measurement of ether phospholipids in human plasma after hydrolysis of plasma with phospholipase A1. Pract Lab Med 2018;10:44–51.

[82] Lee DY, Kind T, Yoon Y-R, et al. Comparative evaluation of extraction methods for simultaneous mass-spectrometric analysis of complex lipids and primary metabolites from human blood plasma. Anal Bioanal Chem 2014;406:7275–7286.

[83] Mung D, Li L. Development of chemical isotope labeling LC-MS for milk metabolomics: Comprehensive and quantitative profiling of the amine/ phenol submetabolome. Anal Chem 2017;89:4435–4443.

[84] Satomi Y, Hirayama M, Kobayashi H. One-step lipid extraction for plasma lipidomics analysis by liquid chromatography mass spectrometry. J Chromatogr B 2017;1062:93–100.

[85] Kiełbowicz G, Micek P, Wawrzeńczyk C. A new liquid chromatography method with charge aerosol detector (CAD) for the determination of phospholipid classes. Application to milk phospholipids. Talanta 2013;105:28–33.

[86] Aoyagi R, Ikeda K, Isobe Y, et al. Comprehensive analyses of oxidized phospholipids using a measured MS/MS spectra library. J Lipid Res 2017;58:2229–2237.

[87] Spickett CM, Rennie N, Winter H, et al. Detection of phospholipid oxidation in oxidatively stressed cells by reversed-phase HPLC coupled with positive-ionization electroscopy MS. Biochem J 2001;355:449–457.

[88] Haller E, Stübiger G, Lafitt D, et al. Chemical recognition of oxidation-specific epitopes in low-density lipoproteins by a nanoparticle based concept for trapping, enrichment, and liquid chromatography-tandem mass spectrometry analysis of oxidative stress biomarkers. Anal Chem 2014;86:9954−9961.

[89] Birjandi AP, Mirnaghi FS, Bojko B, et al. Application of solid phase microextraction for quantitation of polyunsaturated fatty acids in biological fluids. Anal Chem 2014;86:12022–12029.

[90] Milojković-Opsenica D, Andri F. High performance thin-layer chromatography. In: Green Chromatographic Techniques: Separation and Purification of Organic and Inorganic Analytes. 2013. .

[91] Handloser D, Widmer V, Reich E. Separation of phospholipids by HPTLC–An investigation of important parameters. J Liq Chromatogr Relat Technol 2008;31:1857–1870.

[92] Rabel F, Sherma J. Review of the state of the art of preparative thin-layer chromatography. J Liq Chromatogr Relat Technol 2017;40:165–176.

[93] Palusinska-Szysz M, Kania M, Turska-Szewczuk A, et al. Identification of unusual phospholipid fatty acyl compositions of Acanthamoeba castellanii. PLoS One 2014;9:e101243.

[94] Xu G, Waki H, Kon K, et al. Thin-layer chromatography of phospholipids and their lyso forms : application to determination of extracts from rat hippocampal CA1 region. Microchem J 1996;53:29–33.

[95] Tanaka T, Kassai A, Ohmoto M, et al. Quantification of phosphatidic acid in foodstuffs using a thin-layer-chromatography-imaging technique. J Agric Food Chem 2012;60:4156–4161.

[96] Fuchs B, Süß R, Teuber K, et al. Lipid analysis by thin-layer chromatography — A review of the current state. J Chromatogr A 2011;1218:2754–2774.

[97] Dyńska-Kukulska K, Ciesielski W, Zakrzewski R. The use of a new, modified Dittmer-Lester spray reagent for phospholipid determination by the TLC image analysis technique. Biomed Chromatohraphy 2013;27:458–465.

[98] Kriska T, Girotti AW. Separation and quantitation of peroxidized phospholipids using high-performance thin-layer chromatography with tetramethyl-p-phenylenediamine detection. Anal Biochem 2004;327:97–106.

[99] Parchem K, Kusznierewicz B, Chmiel T, et al. Profiling and qualitative assessment of enzymatically and thermally oxidized egg yolk phospholipids using a two-step HPLC protocol. J Am Oil Chem Soc 2019;96:693–706.

[100] Helmerich G, Koehler P. Comparison of methods for the quantitative determination of phospholipids in lecithins and flour improvers. J Agric Food Chem 2003;51:6645–6651.

[101] Dittmer JC, Lester RL. A simple, specific spray for the detection of phospholipids on thin-layer chromatograms. J Lipid Res 1964;5:126–127.

[102] Stillwell W. Chapter 13 - Membrane Reconstitution. In: An Introduction to Biological Membranes (Second Edition). Elsevier; 2016. p. 273–312.

[103] Rhee KS, Del Rosario RR, Dugan JR. Determination of plasmalogens after treating with a 2,4-dinitrophenylhydrazine-phosphoric acid reagent. Lipids 1967;2:334–338.

[104] Fuchs B. Analytical methods for (oxidized) plasmalogens – methodological aspects and applications. Free Radic Res 2014;49:599–617.

[105] Friedman P, Ho S, Steinberg D, et al. Correlation of antiphospholipid antibody recognition with the structure of synthetic oxidized phospholipids. J Biol Chem 2002;277:7010–7020.

[106] Lisa M, Cifkova E, Holcapek M. Lipidomic profiling of biological tissues using off-line two-dimensional high-performance liquid chromatography-mass spectrometry. J Chromatogr A 2011;1218:5146–5156.

[107] Dugo P, Fawzy N, Cichello F, et al. Stop-flow comprehensive two-dimensional liquid chromatography combined with mass spectrometric detection for phospholipid analysis. J Chromatogr A 2013;1278:46–53.

[108] Kim J, Minkler PE, Salomon RG, et al. Cardiolipin : characterization of distinct oxidized molecular species. J Lipid Res 2011;52:125–135.

[109] Jia L, Wang C, Kong H, et al. Plasma phospholipid metabolic profiling and biomarkers of mouse IgA nephropathy. Metabolomics 2006;2:95–104.

[110] Kim J, Hoppel CL. Comprehensive approach to the quantitative analysis of mitochondrial phospholipids by HPLC-MS. J Chromatogr B Anal Technol Biomed Life Sci 2013;912:105–114.

[111] Buré C, Ayciriex S, Testet E, et al. A single run LC-MS/MS method for phospholipidomics. Anal Bioanal Chem 2013;405:203–213.

[112] Anesi A, Guella G. A fast liquid chromatography-mass spectrometry methodology for membrane lipid profiling through hydrophilic interaction liquid chromatography. J Chromatogr A 2015;1384:44–52.

[113] Buszewski B, Noga S. Hydrophilic interaction liquid chromatography (HILIC)-a powerful separation technique. Anal Bioanal Chem 2012;402:231–247.

[114] Gama MR, da Costa Silva RG, Collins CH, et al. Hydrophilic interaction chromatography. Trends Anal Chem 2012;37:48–60.

[115] Schwalbe-Herrmann M, Willmann J, Leibfritz D. Separation of phospholipid classes by hydrophilic interaction chromatography detected by electrospray ionization mass spectrometry. J Chromatogr A 2010;1217:5179–5183.

[116] Cifkova E, Holcapek M, Lisa M, et al. Nontargeted quantitation of lipid classes using hydrophilic interaction liquid chromatography-electrospray ionization mass spectrometry with single internal standard and response factor approach. Anal Chem 2012;84:10064–10070.

[117] Cifkove E, Holcapek M, Lisa M. Nontargeted lipidomic characterization of porcine organs using hydrophilic interaction liquid chromatography and off-line two-dimensional liquid chromatography-electrospray ionization mass spectrometry. Lipids 2013;48:915–928.

[118] Holčapek M, Jirásko R, Lísa M. Recent developments in liquid chromatography – mass spectrometry and related techniques. J Chromatogr A 2012;1259:3–15.

[119] Kong P, Lehmann MJ, Helms JB, et al. Lipid analysis of Eimeria sporozoites reveals exclusive phospholipids, a phylogenetic mosaic of endogenous synthesis, and a host-independent lifestyle. Cell Discov 2018;4:24.

[120] Lísa M, Holčapek M. High-throughput and comprehensive lipidomic analysis using ultrahigh-performance supercritical fluid chromatography − mass spectrometry. Anal Chem 2015;87:7187–7195.

[121] Caudron E, Zhou JY, Chaminade P, et al. Fluorescence probe assisted post-column detection for lipid analysis in microbore-LC. J Chromatogr A 2005;1072:149–157.

[122] Milne GL, Porter NA. Separation and identification of phospholipid peroxidation products. Lipids 2001;36:1265–1275.

[123] Sala P, Pötz S, Brunner M, et al. Determination of oxidized phosphatidylcholines by hydrophilic interaction liquid chromatography coupled to Fourier transform mass spectrometry. Int J Mol Sci 2015;8351–8363.

[124] Stoll DR, Carr PW. Two-dimensional liquid chromatography: A state of the art tutorial. Anal Chem 2017;89:519–531.

[125] Carr PW, Stoll DR. Two-dimensional liquid chromatography: Principles, practical implementation and applications. Agilent Technical Note; 2015.

[126] Nie H, Liu R, Yang Y, et al. Lipid profiling of rat peritoneal surface layers by online normal- and reversed-phase 2D LC QToF-MS. J Lipid Res 2010;51:2833–2844.

[127] Tranchida PQ, Donato P, Cacciola F, et al. Potential of comprehensive chromatography in food analysis. Trends Anal Chem 2013;52:186–205.

[128] Ostrowska J, Skrzydlewska E, Figaszewski ZA. Isolation and analysis of phospholipids. Chem Analityczna 2000;45:613–629.

[129] Restuccia D, Spizzirri UG, Puoci F, et al. Determination of phospholipids in food samples. Food Rev Int 2012;28:1–46.

[130] Ibusuki D, Nakagawa K, Asai A, et al. Preparation of pure lipid hydroperoxides. J Lipid Res 2008;49:2668–2677.

[131] Butler O'Connor ES, Mazerik JN, Cruff JP, et al. Lipoxygenase-catalyzed phospholipid peroxidation: Preparation, purification, and characterization of phosphatidylinositol peroxides. In: Uppu RM, Murthy SN, Pryor WA, et al., editors. Free Radicals and

Antioxidant Protocols. Methods in Molecular Biology (Methods and Protocols). 2nd ed. New York: Humana Press; 2010. p. 387–401.

[132] Milne GL, Porter NA. Separation and identification of phospholipid peroxidation products. Lipids 2001;36:1265–1275.

[133] Schweikart F, Hulthe G. HPLC–UV–MS Analysis: A Source for Severe Oxidation Artifacts. Anal Chem 2019;8–11.

[134] Ibrahim H, Caudron E, Kasselouri A, et al. Interest of fluorescence derivatization and fluorescence probe assisted post-column detection of phospholipids: A short review. Molecules 2010;15:352–373.

[135] Ouhazza M, Sioufii AM. Liquid chromatography analysis of some phospholipids with fluorescence detection. Analusis 1992;20:185–188.

[136] Ramos RG, Libong D, Rakotomanga M, et al. Comparison between charged aerosol detection and light scattering detection for the analysis of Leishmania membrane phospholipids. J Chromatogr A 2008;1209:88–94.

[137] Kiełbowicz G, Trziszka T, Wawrzeńczyk C. Separation and quantification of phospholipid and neutral lipid classes by HPLC-CAD: Application to egg yolk lipids. J Liq Chromatogr Relat Technol 2015;38:898–903.

[138] Han X, Gross RW. Global analyses of cellular lipidomes directly from crude extracts of biological samples by ESI mass spectrometry: a bridge to lipidomics. J Lipid Res 2003;44:1071–1079.

[139] Hou W, Zhou H, Elisma F, et al. Technological developments in lipidomics. Briefings Funct Genomics Proteomics 2008;7:395–409.

[140] Schröter J, Süß R, Schiller J. MALDI-TOF MS to monitor the kinetics of phospholipase A2-digestion of oxidized phospholipids. Methods 2016;104:41–47.

[141] Schröter J, Griesinger H, Reuß E, et al. Unexpected products of the hypochlorous acid-induced oxidation of oleic acid: A study using high performance thin-layer chromatography-electrospray ionization mass spectrometry. J Chromatogr A 2016;1439:89–96.

[142] Fuchs B, Schiller J, Süß R, et al. A direct and simple method of coupling matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS) to thin-layer chromatography (TLC) for the analysis of phospholipids from egg yolk. Anal Bioanal Chem 2007;389:827–834.

[143] Schiller J, Süß R, Fuchs B, et al. Combined application of TLC and matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry (MALDI-TOF MS) to phospholipid analysis of brain. Chromatographia 2003;57:297–302.

[144] Fuchs B, Schiller J, Süß R, et al. Capabilities and disadvantages of combined matrix-assisted laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and high-performance thin-layer chromatography (HPTLC): Analysis of egg yolk lipids. JPC - J Planar Chromatogr - Mod TLC 2009;22:35–42.

[145] Domingues MRM, Reis A, Domingues P. Mass spectrometry analysis of oxidized phospholipids. 2008;156:1–12.

[146] Reis A, Domingues P, Domingues MRM. Structural motifs in primary oxidation products of palmitoyl-arachidonoyl-phosphatidylcholines by LC-MS/MS. J Mass Spectrom 2013;48:1207–1216.

[147] Reis A, Domingues MRM, Amado FML, et al. Radical peroxidation of palmitoyl-lineloyl-glycerophosphocholine liposomes: Identification of long-chain oxidised products by liquid chromatography – tandem mass spectrometry. J Chromatogr B 2007;855:186–199.

[148] Deeley JM, Thomas MC, Truscott RJW, et al. Identification of abundant alkyl ether glycerophospholipids in the human lens by tandem mass spectrometry techniques. Anal Chem 2009;81:1920–1930.

[149] Ali AH, Zou X, Lu J, et al. Identification of phospholipids classes and molecular species in different types of egg yolk by using UPLC-Q-TOF-MS. Food Chem 2017;221:58–66.

[150] Reis A, Domingues P, Ferrer-Correia AJV, et al. Tandem spectrometry of intact oxidation products of diacylphosphatidylcholines: Evidence for the occurrence of the

oxidation of the phosphocholine head and differentiation of isomers. J Mass Spectrom 2004;39:1513–1522.

[151] Rathahao-Paris E, Alves S, Junot C, et al. High resolution mass spectrometry for structural identification of metabolites in metabolomics. Metabolomics 2015;12:10.

[152] Baker PRS, Armando AM, Campbell JL, et al. Three-dimensional enhanced lipidomics analysis combining UPLC, differential ion mobility spectrometry, and mass spectrometric separation strategies. J Lipid Res 2014;55:2432–2442.

[153] Ni Z, Milic I, Fedorova M. Identification of carbonylated lipids from different phospholipid classes by shotgun and LC-MS lipidomics. Anal Bioanal Chem 2015;407:5161–5173.

[154] Nakanishi H, Iida Y, Shimizu T, et al. Analysis of oxidized phosphatidylcholines as markers for oxidative stress, using multiple reaction monitoring with theoretically expanded data sets with reversed-phase liquid chromatography/tandem mass spectrometry. J Chromatogr B 2009;877:1366–1374.

[155] Spickett CM, Pitt AR. Oxidative lipidomics coming of age: Advances in analysis of oxidized phospholipids in physiology and pathology. Antioxid Redox Signal 2015;22:1646–1666.

[156] Kliman M, May JC, Mclean JA. Lipid analysis and lipidomics by structurally selective ion mobility-mass spectrometry. BBA - Mol Cell Biol Lipids 2011;1811:935–945.

[157] Groessl M, Graf S, Taylor A, et al. Separation of isomeric lipids by ion mobility-time of flight mass spectrometry. 2015;1–4.

[158] Jackson SN, Ugarov M, Post JD, et al. A Study of phospholipids by Ion Mobility TOFMS. J Am Soc Mass Spectrom 2008;19:1655–1662.

[159] Orešič M. Bioinformatics and computational approaches applicable to lipidomics. Eur J Lipid Sci Technol 2009;111:99–106.

[160] Checa A, Bedia C, Jaumot J. Lipidomic data analysis: Tutorial, practical guidelines and applications. Anal Chim Acta 2015;885:1–16.

[161] Dalmau N, Bedia C, Tauler R. Validation of the Regions of Interest Multivariate Curve Resolution (ROIMCR) procedure for untargeted LC-MS lipidomic analysis. Anal Chim Acta 2018;1025:80–91.

[162] Hartler J, Tharakan R, Köfeler HC, et al. Bioinformatics tools and challenges in structural analysis of lipidomics MS/MS data. Brief Bioinform 2012;14:375–390.

[163] Wang M, Wang C, Han RH, et al. Novel advances in shotgun lipidomics for biology and medicine. Prog Lipid Res 2016;61:83–108.

[164] Kind T, Liu K-H, Lee DY, et al. LipidBlast - in-silico tandem mass spectrometry database for lipid identification. Nat Methods 2013;10:755–758.

[165] Koelmel JP, Kroeger NM, Ulmer CZ, et al. LipidMatch: an automated workflow for rule-based lipid identification using untargeted high-resolution tandem mass spectrometry data. BMC Bioinformatics 2017;18:1–11.

[166] Ni Z, Angelidou G, Hoffmann R, et al. LPPtiger software for lipidome-specific prediction and identification of oxidized phospholipids from LC-MS datasets. Sci Rep 2017;1–14.

[167] Kochen MA, Chambers MC, Holman JD, et al. Greazy: open-source software for automated phospholipid MS/MS identification. Anal Chem 2016;88:5733–5741.

[168] Ejsing CS, Duchoslav E, Sampaio J, et al. Automated identification and quantification of glycerophospholipid molecular species by multiple precursor ion scanning. Anal Chem 2006;78:6202–6214.

[169] Herzog R, Schuhmann K, Schwudke D, et al. LipidXplorer: A software for consensual cross-platform lipidomics. PLoS One 2012;7:e2985.

[170] Gode D, Volmer DA. Lipid imaging by mass spectrometry – a review. Analyst 2013;138:1289–1315.

[171] Bednařík A, Machálková M, Moskovets E, et al. MALDI MS imaging at acquisition rates exceeding 100 pixels per second. J Am Soc Mass Spectrom 2018;1–10.

[172] Shimizu Y, Satou M, Hayashi K, et al. Matrix-assisted laser desorption/ionization imaging mass spectrometry reveals changes of phospholipid distribution in induced

pluripotent stem cell colony differentiation. Anal Bioanal Chem 2017;409:1007–1016.

[173] Hong JH, Kang JW, Kim DK, et al. Global changes of phospholipids identifi ed by MALDI imaging mass spectrometry in a mouse model of Alzheimer's disease. J Lipid Res 2016;57:36–45.

[174] Eberlin LS, Tibshirani RJ, Zhang J, et al. Molecular assessment of surgical-resection margins of gastric cancer by mass-spectrometric imaging. Proc Natl Acad Sci 2014;111:2436–2441.

[175] Shimma S, Sugiura Y, Hayasaka T, et al. MALDI-based imaging mass spectrometry revealed abnormal distribution of phospholipids in colon cancer liver metastasis. J Chromatogr B 2007;855:98–103.

[176] Eberlin LS, Norton I, Dill AL, et al. Classifying human brain tumors by lipid imaging with mass spectrometry. Cancer Res 2012;72:645–655.

[177] Mao X, He J, Li T, et al. Application of imaging mass spectrometry for the molecular diagnosis of human breast tumors. Sci Rep 2016;6:21043.

[178] Stutts WL, Menger RF, Kiss A, et al. Characterization of phosphatidylcholine oxidation products by MALDI MSn. Anal Chem 2013;85:11410–11419.

[179] Patterson NH, Chaurand P. Monitoring time-dependent degradation of phospholipids in sectioned tissues by MALDI imaging mass spectrometry. J Mass Spectrom 2014;49:622–627.

[180] Maulucci G, Cohen O, Daniel B, et al. Fatty acid-related modulations of membrane fluidity in cells : detection and implications. Free Radic Res 2016;50:S40–S50.

[181] Cohen G, Riahi Y, Shamni O, et al. Role of lipid peroxidation and PPAR-d in amplifying glucose-stimulated insulin secretion. Diabetes 2011;60:2830–2842.

[182] Cohen G, Shamni O, Avrahami Y, et al. Beta cell response to nutrient overload involves phospholipid remodelling and lipid peroxidation. Diabetologia 2015;58:1333–1343.

[183] Maulucci G, Di Giacinto F, De Angelis C, et al. Real time quantitative analysis of lipid storage and lipolysis pathways by confocal spectral imaging of intracellular micropolarity. BBA - Mol Cell Biol Lipids 2018;1863:783–793.

[184] Drummen GPC, van Liebergen LCM, Op den Kamp JAF, et al. C11-BODIPY(581/591), an oxidation-sensitive fluorescent lipid peroxidation probe: (micro)spectroscopic characterization and validation of methodology. Free Radic Biol Med 2002;33:473–490.

[185] Gibbons E, Nelson J, Anderson L, et al. Role of membrane oxidation in controlling the activity of human group IIa secretory phospholipase A 2 toward apoptotic lymphoma cells. BBA - Biomembr 2013;1828:670–676.

[186] Yamanaka K, Saito Y, Sakiyama J, et al. A novel fluorescent probe with high sensitivity and selective detection of lipid hydroperoxides in cells. RSC Adv 2012;2:7894–7900.

[187] Kagan VE, Mao G, Qu F, et al. Oxidized arachidonic/adrenic phosphatidylethanolamines navigate cells to ferroptosis. Nat Chem Biol 2017;13:81–90.

Figure captions:

Figure 1: Number of publications on lipid-related topics from 1950 to 2015. Reprinted with permission from [25].

Figure 2: Chemical structures of phospholipids.

Figure 3: The extraction efficiency of individual lipid classes using five different solvent extraction protocols: Folch (1), Bligh and Dyer (2), acidified Bligh and Dyer (3), MTBE/ methanol (4) and hexane/2-propanole (5). The graphs shown the intensity in counts per second (cps). Abbreviations: TAG – triacylglycerol; CE – cholesterol ester; PC – phosphatidylcholine; SM – sphingomyelin; PE – phosphatidylethanolamine; Cer – ceramide; FA – fatty acid; PI – phosphatidylinositol; LacCer – lactosyl ceramide; LAA – lipoamino acid; CS – cholesterol sulphate; Lyso-PC – lysophosphatidylcholine. Reprinted with permission from [51].

Figure 4: 1D HPTLC chromatogram of soy lecithin (left) and HT-29 cell (right) phospholipids separated on silica gel plate using a mobile phase composed of chloroform/methanol/acetic

acid/acetone/water (35:25:4:14:2 *v/v/v/v/v*) and visualized with molybdenium reagent. The soy lecithin was dissolved in chloroform at a concentration of 5 mg/mL. The lipid fraction extracted from HT-29 cells using Bligh and Dyer method was dissolved in chloroform/methanol mixture (2:1 *v/v*) at a concentration of 20 mg/mL. The samples were applied onto TLC plate in a volume of 40 μL as 8 mm bands. Abbreviations: LPCs – lysophosphatidylcholines; SMs – sphingomyelins; PCs – phosphatidylcholines; LPEs – lysophosphatidylethanolamines; PSs – phosphatidylserines; PIs – phosphatidylinositols; PEs – phosphatidylethanolamines; PAs – glycerophosphates; PGs – phosphatidylglycerols; un – unknown.

Figure 5: 1D HPTLC chromatograms of hen egg yolk PL fraction extracted with methanol: (a and b), profiles of native PL classes visualized with acidic copper sulfate solution and molybdenum reagent, respectively; (c) DMP-visualized profiles of native (0 min) and enzymatically oxidized PL classes after 10, 20, 30, 60, 90, and 120 min of the soybean LOX 1-catalyzed reaction. Abbreviations: LPCs, lysophosphatidylcholines; SMs, sphingomyelins; PCs, phosphatidylcholines; LPEs, lysophosphatidylethanolamines; PIs, phosphatidylinositols; PEs, phosphatidylethanolamines; PGs, phosphatidylglycerols; and TAG, triacylglycerols. Reprinted with permission from [99].

Figure 6: HILIC-MS chromatogram of lipid classes represented by standards containing oleic acid on a Spherisorb Si column (250 × 4.6 mm, 5 μm) with a gradient elution using a mobile phase composed of acetonitrile and 5 mM aqueous ammonium acetate and ESI-MS detection in positive (A) and negative (B) ion mode. Abbreviations: TG – triacylglycerol; Chol – cholesterol; CE – cholesteryl ester; FA – fatty acid; PG – phosphatidylglycerol; 2-LPG – 2-lysophosphatidylglycerol; 1-LPG – 1-lysophosphatidylglycerol; PI – phosphatidylinositol; CA – cardiolipins; LPI – lysophosphatidylinositol; pPE – phosphatidylethanolamine plasmalogen; PE – phosphatidylethanolamine; 2-LPE – 2-lysophosphatidylethanolamine; 1-LPE – 1-lysophosphatidylethanolamine; pPC – phosphatidylcholine plasmalogen; PC – phosphatidylcholine; SM – sphingomyelin; 2-LPC – 2-lysophosphatidylcholine; 1-LPC – 1-lysophosphatidylcholine. Reprinted with permission from [106].

Figure 7: Enlargement of the HILIC×RPLC-MS contour plot of the glycerophosphatidylcholine molecular species of plasma (left), along with the corresponding 2D raw data (right). The 1D separation was carried out on an Ascentis Express HILIC column (150 × 2.1 mm, 2.7 μm) with a gradient elution using mobile phase composed of acetonitrile/10 mM aqueous ammonium

formate (90/10) and acetonitrile/methanol/10 mM aqueous ammonium formate (55:35:10). The ²D separation was carried out on an Ascentis Express C18 column (150 × 4.6 mm, 2.7 μm) with a gradient elution using mobile phase composed of isopropanol/10 mM ammonium formate/ tetrohydrofuran (55:30:15) and acetonitrile. Reprinted with permission from [107].

Figure 8: HPLC-CAD chromatogram of the hen egg yolk PLs obtained during the separation in the HILIC mode (A). CAD-HPLC chromatograms obtained during separation in the RP mode for oxidized and non-oxidized PE (B) and PC (C) species formed after 60 min of soybean LOX-1 catalyzed reaction. HPLC-DAD chromatograms obtained during separation in the RP mode for oxidized PE (D) PC (E) species formed after 60 min of soybean LOX-1 catalyzed reaction. Abbreviations: TAGs – triacylglycerols; FAs – fatty acids; PGs – phosphatidylglycerols; PIs – phosphatidylinositols; PEs – phosphatidylethanolamines; LPEs – lysophosphatidylethanolamines; PCs – phosphatidylcholines; SMs – sphingomyelins; LPCs – lysophosphatidylcholines. Reprinted with permission from [99].

Figure 9: Separation of PL classes by HILIC-UPLC. Multiplex analysis of mixture of PL class standards (A) and human serum (B) using PIS and NLS in positive and negative ion mode. Top panel shows HILIC chromatographic separation and mass spectrometric detection of PL classes by either precursor *m/z* 184, 241, 264 or neutral loss *m/z* 141, 172, 185, 98. Middle panel shows NLS *m/z* 141 in positive ion mode specific for PE and LPE. Bottom panel shows summed mass spectral (MS) data from NLS *m/z* 141 in positive ion mode shown indicated in grey above. Inset: magnification from *m/z* 762 to *m/z* 774. Abbreviations: PG – phosphatidylglycerol; PI – phosphatidylinositol; PE – phosphatidylethanolamine; LPE – 1-lysophosphatidylethanolamine; PC – phosphatidylcholine; PA – phosphatidic acid; SM – sphingomyelin; LPC – lysophosphatidylcholine. Reprinted with permission from [152].

Figure 10: Overlay of two 2D MALDI-IMS-MS plots of rat brain phospholipids. The analysis was carried out directly from the brain tissue, on which matrix solution (2,5-dihydroxybenzoic acid) without cesium (blue area) and with cesium (red area) was deposited. Reprinted with permission from [158].

Figure 11: AFAI-MSI of samples of low-grade breast ductal carcinoma *in situ* (A and C) and high-grade breast invasive ductal carcinoma (B and D). Positive ion mode AFAI-MSI ion images of two samples showing the distribution of *m/z* 706.5 corresponding to PC(30:0) (A1, B1), *m/z* 718.6 corresponding to PC(32:0) or PE(35:0) (A2, B2), *m/z* 724.5 corresponding to PE(34:1)

(A3, B3) and *m/z* 730.5 corresponding to PC(32:2) (A4, B4). Negative ion mode AFAI-MSI ion images of sample two samples showing the distribution of *m/z* 295.2, corresponding to FA(18:2) (C1, D1), *m/z* 311.2 corresponding to octadecanoids (C2, D2), *m/z* 327.2 corresponding to FA(22:6) (C3, D3) and *m/z* 329.2 corresponding to FA(22:5) (C4, D4). Lower magnification images with expanded views of an adjacent hematoxylin and eosin (H&E) stained section are shown in (E). Reprinted with permission from [177].

Figure 12: Effects of nutrient overload on membrane fluidity in insulin secreting beta cells. INS-1E cells expressing the m-cherry protein in insulin granules were used, as explained in [180]. (A) The cells were grown at 11 mM glucose (G11) and treated with 500 µM palmitic acid. Images were collected before the addition of palmitic acid and 16 h later. The upper images are of Laurden emission and the lower images depict m-cherry emission (labelling insulin granules). Membrane fluidity is represented in terms of ratio of emission intensities by using the Generalized Polarization (GP) value. The GP ranges from -1 (very fluid-purple) to +1 (very gel-like-orange). M-cherry labels insulin granules, which have spherical shapes of about 0.5 – 1 µm diameter. (B) GP values of plasma membrane in cells incubated with increasing glucose concentration for 32 h. Scale bar is 10 µm. Reprinted with permission from [180].

Table 1. Comparison of sample preparation techniques for lipid analysis
with particular reference to phospholipids and their oxidized derivatives

| Technique Parameter | Chloroform-based liquid-liquid extraction (Folch's method and Bligh and Dyer method) | Chloroform-free liquid-liquid extraction | | Ultrasound-assisted extraction (UAE) | Microwave-assisted extraction (MAE) | Precipitation methods (PPT) | Combination of LLE/ PPT with solid phase extraction (SPE) | Direct immersion solid phase micro-extraction (DI-SPME) |
		MTBE method	BUME method					
Application example	Human blood plasma	Human blood plasma	Human blood plasma	Human blood plasma	Freeze-dried yeast cells	Human blood plasma	Human blood plasma	Human breast milk Cell homogenate
Sample volume/ mass	50 μL	20–50 μL	≥75 μL	30 μL	10 mg	200 μL	20-50 μL used to LLE or PPT (500 μL of lipid extract)	1 mL
Solvent system used	Chloroform; methanol; 0.9 % *w/v* aqueous NaCl solution (or water)	MTBE; methanol; water	Butanol; methanol; heptane; ethyl acetate 1% *v/v* aqueous acetic acid	e.g., MTBE; methanol; water	e.g., chloroform; methanol	2-propanol	Methanol; 0.2 % formic acid; 2-propanol	2-propanol; methanol (for lipid desorption)
Sample amount to extractant volume ratio	1:20 *v/v**	1:100 *v/v**	1:12 *v/v**	1:5 to 1:25 *v/v* (based on MTBE volume)	7 mL of the solvent mixture per 10 mg of the sample	1:3 to 1:5 *v/v*	2.5 mL of solvents per sample used during SPE	The volume of solvent required for the lipid desorption is 100 μL – 1 mL
Isolation time	Extraction time: 40 min**	Extraction time: 70 min** (Simultaneous extraction of 96 samples is possible at this time using 96-well robots)	Extraction time: 35 min** (Simultaneous extraction of 96 samples is possible at this time using 96-well robots)	Extraction time: 30 min**	Extraction time: 16 min**	Extraction time: overnight	Extraction time depended on method used Solid phase extraction: e.g., 10 samples in 30 min using spin columns Solvent evaporation	Pre-condition of a fiber: 20 – 30 min Lipid absorption on fiber: 5 – 90 min Lipid desorption: 5 - 60 min (Simultaneous lipid isolation from 96 samples is possible at this time using 96-well robots)

Cost	Inexpensive apparatus Inexpensive solvent used Long working time	Inexpensive solvent used (Higher cost to purchase the automatic robots)	Inexpensive solvent used (Higher cost to purchase the automatic robots)	Inexpensive solvent used Higher cost to purchase the system	Inexpensive solvent used Higher cost to purchase the system	Inexpensive solvent used (5 times cheaper than LLE)	Inexpensive solvent used Expensive cartridges	Inexpensive solvent used Expensive SPME fibers
Lipid analysis technique used	LC-MS LC-MS/MS	LC-MS LC-MS/MS	LC-ELSD LC-MS	LC-MS	LC-CAD	LC-MS	LC-MS/MS MALDI-MS/MS	LC-MS/MS
Recovery	Recovery values of PC for Folch, acidified Bligh and Dyer and Bligh and Dyer methods is around 100, 90 and 75 %, respectively.	Similar recovery values for PC and higher for PI compared to Folch's method	Similar recovery values for PC, PE, PA, LPC and SM and higher for PA, PG, PS, PG and LPA compared to Folch's method	Higher recovery values for SM classes compared to Bligh and Dyer method and for PE and SM compared to previous reported MTBE method Lower recovery value for PC compared to Bligh and Dyer method and previous reported MTBE method	The recoveries were estimated for cholesterol (92 %), TAG (95 %) and FFA (92 %)	The recovery values for PC, PE, PG, PS, LPC and SM were in the range of 60-90 %	The recovery value for oxPC is 67.5 %	The recoveries value for PG, PE, PC, LPC and SM in the range of 55 – 70 %
Lipidome coverage	High lipid coverage for Folch's method and acidified Bligh and Dyer	Lipid coverage comparable to chloroform-based LLE	Lipid coverage comparable to Folch's method	Higher overall number of lipids identified compared to Bligh and Dyer method and previous reported MTBE method	Lipid coverage comparable to chloroform based LLE	Broad lipid coverage (LPC, LPE, PC, PG, PS, SM)	This technique is aimed at isolation of specific lipid species e.g., oxPCs	SPME enables the isolation of broader range of lipid categories/ classes compared to Folch's method, however, the latter allows better coverage of high-abundant membrane PL (PCs and PEs)
Repeatability/reproducibility	Standard deviations below 5 %	Standard deviations of the mean	Standard deviation	High repeatability compared	Higher repeatability compared to	Coefficient of variation below 20 %	Coefficient of variation is 9.6 %	Relative standard deviations

	for Folch's and Bligh and Dyer methods	values below 6 %	values below 8 %	to Bligh and Dyer method and previous reported MTBE (Relative standard deviations below 5.55 %)	chloroform-based LLE (Intraday and interday relative standard deviations in the range of 0.63−6 % and 4−12%, respectively			below 14 % for tested PL classes
Potential for automation	Low	High	High	Low	Low	High	High	High
Remarks	Fire, health and environmental hazards Lipid-containing organic phase forms the bottom layer during phase separation	Lipid-containing organic phase forms the upper layer during phase separation	Lipid-containing organic phase forms the upper layer during phase separation	Frequencies from 20 kHz to several GHz are used.	During the extraction temperature increases, which can lead to the breakdown of thermolabile analytes	The protein removal efficiency is 99 % Technique requires little labour input	Prior sample preparation using LLE or PPT is required The columns can be reused, which significantly reduces costs.	DI-SPME is an equilibrium technique
Reference	[50–52]	[50,51,53–55]	[56–58]	[59,60]	[61]	[62]	[63,64]	[48,49]

* - Based on total extractant volume including re-extraction

** - The time of centrifugation, lipid-containing phase transfer, (potential re-extraction) and solvent evaporation should be added.

Table 2. Derivatization reagents used to visualize PL following TLC separation

Derivatization reagent	Specificity	Analyte appearance	Reference
Acetic aqueous solution of copper (II) sulphate	Organic compounds	Black spots/bands	[100]
Primulin solution	Lipid compounds	Fluorescente spots/bands ($\lambda^{ex/em}$=312/605 nm	[95]
Dittmer-Lester reagent (with further modifications)	PLs	Blue spots/bands on a white background	[97,101]
Malachite green reagent	PLs	Bright green spots/bands on a orange background	[102]
2,4-dinitrophenylhydrazine	Plasmalogens	Yellow-orange spots/bands on a white background	[103,104]
Dragendorff reagent	Choline-containing PLs (LPCs, PCs, SMs)	Orange-red spots/bands	[93]
Ninhydrin	Free amino group-containing PLs (LPEs, LPSs, PEs,PSs)	Red-violet spots/bands	[93]
N,N-dimethyl-*p*-phenylenediamine dihydrochloride	PL hydroperoxides	Purple spots/bands	[69]
N,N,N′,N′-tetramethyl-*p*-phenylenediamine	PL hydroperoxides	Purple spots/bands	[98]
Schiff's reagent	PLs with aldehyde moiety	Red-violet spots/bands	[105]

24