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REVIEW

Lipidomic Analysis: From Archaea to Mammals

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Abstract Lipids are among the most important organic compounds found in all living cells, from primitive archaebacteria to flowering plants or mammalian cells. They form part of cell walls and constitute cell storage material. Their biosynthesis and metabolism play key roles in faraway topics such as biofuel production (third-generation biofuels produced by microorganisms, e.g. algae) and human diseases such as adrenoleukodystrophy, Zellweger syndrome, or Refsum disease. Current lipidomic analysis requires fast and accurate processing of samples and especially their characterization. Because the number of possible lipids and, more specifically, molecular species of lipids is of the order of hundreds to thousands, it is necessary to process huge amounts of data in a short time. There are two basic approaches to lipidomic analysis: shotgun and liquid chromatography–mass spectometry. Both methods have their pros and cons. This review deals with lipidomics not according to the type of ionization or the lipid classes analyzed but according to the types of samples (organisms) under study. Thus, it is divided into lipidomic analysis of archaebacteria, bacteria, yeast, fungi, algae, plants, and animals.

Keywords Algae · Animals · Archaea · Bacteria · Flower plants · Lipidomics · Mycobacteria · Yeast

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Abbreviations

^{1}H NMR	proton nuclear magnetic resonance
ALA	alpha-linolenic acid (18:3n-3)
APCI	atmospheric pressure chemical ionization
APPI	atmospheric pressure photoionization
Cer	ceramide
DAG	diacylglycerol
DESI	desorption electrospray ionization
DGCC	diacylglycerylcarboxyhydroxymethylcholine
DGDG	digalactosyldiacylglycerol
DGTA	diacylglycerylhydroxymethyltrimethylalanine
DGTS	diacylglyceryltrimethylhomoserine
ESI	electrospray ionization
FA	fatty acid
GA	gadoleic acid
GDGT	glycerol dibiphytanyl glycerol tetraether membrane lipid
GlcCer	glucosylceramide
GlcInsPCer	glycosylinositolphosphoceramide
HILIC	hydrophilic interaction chromatography
HPLC	high-performance liquid chromatography
HPTLC	high-performance thin layer chromatography
HR	high resolution
InsPCer	inositolphosphoceramide
LNA	linoleic acid
M(InsP)$_2$Cer	mannosyl-diinositolphosphoceramide
MALDI	matrix-assisted laser desorption/ionization
MGDG	monogalactosyldiacylglycerol

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MInsPCer	mannosyl-inositolphosphoceramide
MS	mass spectrometry
NARP	nonaqueous reversed-phase
NP	normal-phase
OLA	oleic acid
PAM	palmitic acid
PCA	principal component analysis
Ptd_2Gro	cardiolipin
PtdCho	phosphatidylcholine
PtdEtn	phosphatidylethanolamine
PtdGro	phosphatidylglycerol
PtdGroP	phosphatidylglycerol phosphate
PtdIns	phosphatidylinositol
PtdIns-P	phosphatidylinositol phosphate
PtdIns-P_2	phosphatidylinositol diphosphate
PtdIns-P_3	phosphatidylinositol triphosphate
PtdOH	phosphatidic acid
PtdSer	phosphatidylserine
PUFA	polyunsaturated fatty acid
QTOF	quadrupole time-of-flight
RP	reversed phase
SQDG	sulfoquinovosyldiacylglycerol
STA	stearic acid
TAG	triacylglycerol
TLC	thin-layer chromatography
TOF	time-of-flight
UHPLC	ultrahigh-performance liquid chromatography
VLCFA	very-long-chain fatty acid

Introduction

Lipidomics is derived from the word "lipidome," which is a term describing the complete lipid profile of any cells from the primitive archaea to flowering plants and mammals, including humans. It is basically a subset of the word "metabolome," which also includes other biological classes of compounds, that is, proteins, carbohydrates, nucleic acids, and so on. Lipidomics is essentially a technique of investigating lipids (for their definitions see below) using soft ionization techniques of mass spectrometry. Mass spectrometry is the experimental approach that underlies the recent huge development of lipidomics. It was the advent of new techniques, such as electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI), matrix-assisted laser desorption/ionization (MALDI), to name only the main ones, along with the falling prices of devices to more or less reasonable levels, that gave an immense impetus to an unprecedented development of analytical methods to be used in lipid studies.

Lipidomic research involves the identification and quantification of the thousands of cellular lipid molecular species and their interactions with other lipids, proteins, and other cell components. Investigators in lipidomics examine the structures, functions, interactions, and dynamics of cellular lipids and their changes that occur during perturbation of the system.

The definition of lipids is extensive, and it has not yet been completely elucidated what a lipid is and what it is not. A historical cross-section of definitions of lipids is shown, for example, on the web pages (Christie, 2017) or (The American Oil Chemists' Society, 2017). Lipids can be divided into eight categories (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides) containing distinct classes and subclasses of molecules.

In essence, the major component of a lipid is a long, aliphatic, hydrocarbon-type chain, which is, with some exceptions, bound to a polar portion of the molecule, see Fig. 1.

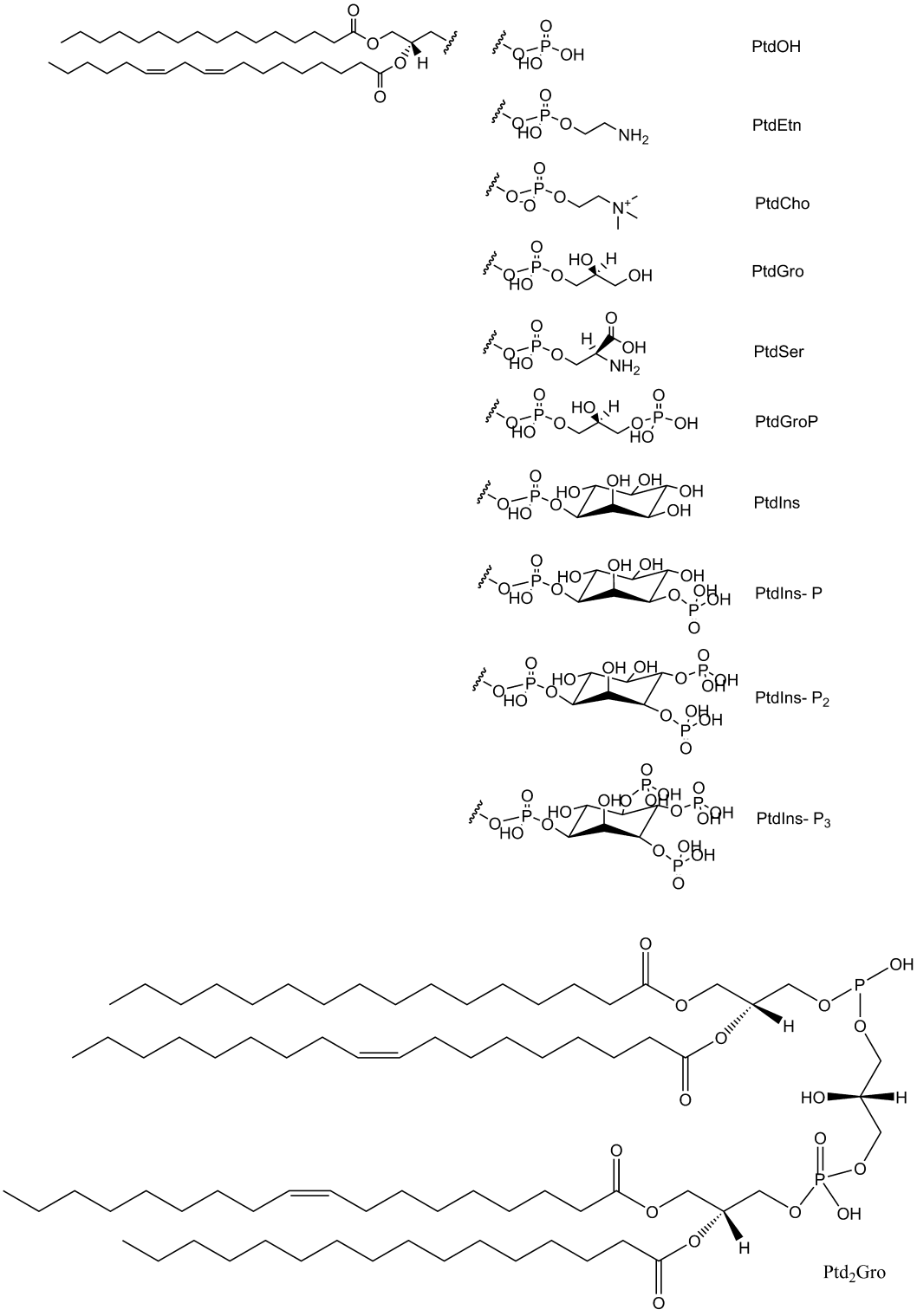
The functions of lipids are as diverse as their structures. The basic functions of lipids include their roles as a source of energy or as building blocks of membranes. Lipids are relatively small hydrophobic molecules, which are good candidates for signaling purposes, for example, in bacteria (Eyster, 2007).

The structural variability of lipids is enormous, and it is not inferior to other classes of compounds studied using metabolomics. As an example, Table 1 shows the number of theoretically possible triacylglycerols (TAG) in a conventional vegetable oil, in this case soya (Daubert, 1949). Of course, not all the theoretically predicted TAG can be identified, which is due to chain preferences depending on the cell type and also to detection limits. Nevertheless, several hundred distinct glycerophospholipid molecular species have been detected in mammalian cells.

Regarding the polar lipids, a very simple cell such as bacterium *Staphylococcus aureus* contains thousands of molecular species of phospholipids and glycolipids (Hewelt-Belka et al., 2014).

In the following, we refrain from the usual division of lipids according to the ionization techniques used for their identification, but divide them according to the studied organisms, from primitive prokaryotic bacteria to multicellular eukaryotic flowering plants and vertebrates (mammals, including humans). We should also mention two basic methods of lipidomics: samples are often infused continuously via a syringe pump into the mass spectrometer (shotgun lipidomics) (Han & Gross, 2005), or a combination of a mass spectrometer with a liquid chromatograph, that is, liquid chromatography–mass spectrometry (LC–MS). Compared to LC–MS, shotgun lipidomics has some advantages, in particular a short analysis time, high throughput, and analysis under a constant concentration of the lipid solution. The fact that a mass spectrum displaying protonated molecules of individual molecular species of a

Fig. 1 Structures of some glycerophospholipids



lipid class of interest can be acquired at a constant concentration of the solution during direct infusion constitutes a major advantage of shotgun lipidomics over LC–MS analysis of lipids. The disadvantages are the impossibility of identifying isobaric molecules (tandem MS can be used for their analysis) and the impossibility of identifying

optical isomers, for instance, the enantiomers of TAG *sn*-STASTAOLA and *sn*-OLASTASTA (OLA oleic acid, STA stearic acid).

Quantification by LC–MS is easy because molecular species are sufficiently separated to allow their peaks to be individually integrated. Calculations in Table 1 allow the theoretical number of molecular species to be calculated from the number of fatty acids present in any sample, depending on the desired level of isomeric structural specificity. Regioisomers are quantified using diacylglycerol-like fragment ions $[DAG]^+$, for instance, $[SO]^+$ against $[SS]^+$, since it is known that, as already noted (Byrdwell, 2005a; Fauconnot, Hau, Aeschlimann, Fay, & Dionisi, 2004; Leskinen, Suomela, & Kallio, 2010; Mottram, 2005), the ratios of the intensities of $[DAG]^+$ ions, that is, $[M\text{-}RCOO]^+$ ions, make it possible to identify the FA in the *sn*-2 position because loss of FA in the *sn*-2 position is assumed to be energetically less favorable than the loss of FA from the *sn*-1 or *sn*-3 position. This procedure can be used only for LC–MS analysis, not for shotgun analysis.

In the following, we chose a rather unconventional division of individual sections. Unlike the chapters in recently published books (Han, 2016; Leray, 2012), the review of different authors (Han, Yang, & Gross, 2012; Wang, Wang, Han, & Han, 2016; Yang et al., 2016), and many subchapters in Byrdwell (2005b), which are always sorted either by the type of ionization techniques or by individual classes of lipids, we chose classification according to the organism under study. We believe that this division is closer to practice, since the analyses are always performed either on individual organisms (or parts of multicellular organisms, e.g. brain, liver, etc.), or on related organisms (e.g. plankton, a mixed culture of microorganisms living in water).

Archaea

Archaea (Archaea, from the Greek ἀρχαῖα, archaia or ancient) form a large group (domain) of prokaryotic single-celled organisms whose independence from other domains of life (bacteria and eukaryotes) was revealed only in 1977 (Garrett & Klenk, 2008). The sizes of the cells of various representatives vary considerably, ranging usually from 0.1 to 15 μm. The structures of their cell membranes and cell walls differ from those of bacteria and eukaryotes, as do the genome and certain metabolic processes. They reproduce by binary fission. Archaea appeared on Earth already 3.5 billion years ago. Although many archaea are found in a range of different environments, today they are primarily known as extremophile organisms, having developed in habitats with extremely high temperatures, extreme pH, or high salt content (Garrett & Klenk, 2008).

Table 1 Number of possible TAG calculated from the number of FA in samples

Description	Number of possible TAG	Number of possible TAG for $y = 11$
Without isomers	$x = (y^3 + 3y^2 + 2y)/6$	286
Without enantiomers	$x = (y^2 + y^3)/2$	726
All isomers	$x = y^3$	1331

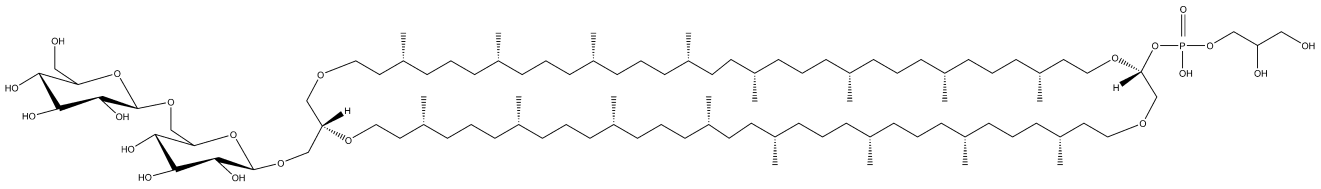
FA, fatty acid; TAG, triacylglycerol.

x is the number of TAG, *y* is the number of FA in TAG.

Archaea have a cytoplasmic membrane surrounded by the cell wall (Thomas, Bardy, & Jarrell, 2001) and are thus structurally similar to Gram-positive bacteria. Unlike their general structure, the chemical structure of archaeal membranes is quite unique and different from that of other forms of life, namely bacteria and eukaryotes (Koga & Morii, 2007). Though cell membranes of all three groups consist of phospholipids that have hydrophilic and hydrophobic parts, archaeal phospholipids are unusual in several respects. They consist of glycerol-ether lipids, while the membranes of bacteria and eukaryotes consist mainly of glycerol-ester lipids (De Rosa, Gambacorta, & Gliozzi, 1986). Ether linkages in archaea are more stable, which may contribute to the fact that archaea are able to live at extreme temperatures and in acidic and alkaline environments (Albers, van de Vossenberg, Driessen, & Konings, 2000). Also the lipid chains exhibit differences: in archaea they consist mainly of isoprene units (Damste, Schouten, Hopmans, van Duin, & Geenevasen, 2002), whereas the main chemical components in other organisms are fatty acids. Isoprenes are branched and, again, more stable at higher temperatures (Koga & Morii, 2005). Archaea differ in the very type of glycerol since they have a different enantiomer (L-glycerol) than all other organisms. It has a different spatial arrangement and this fact points to a different enzyme apparatus in comparison with other life domains (Koga & Morii, 2005). Instead of the normal phospholipid bilayer, only a single-layer membrane is seen some archaea. The tails of phospholipid molecules in the membrane are attached to each other, forming thereby basically a single molecule with two polar ends. Archaea with this type of membranes are clearly more resistant to adverse environments (Hanford & Peeples, 2002). They include, for example, acidophilic archaea of the genus *Ferroplasma*, which are able to withstand low pH (Macalady et al., 2004). The main problem in the analysis of most archaea is their nonculturability. This issue, however, goes far beyond the topic of this chapter. More information can be found, for example, in the paper by Woese, Kandler, and Wheelis (1990).

A combination of thin-layer chromatography (TLC) and MALDI-TOF/MS was used to identify polar lipids of archaeol type (diethers) and caldarchaeol (Fig. 2) lipids

Fig. 2 Polar membrane lipid (caldarchaeol, termed hexose2 PtdGro-T) of *Pyrococcus furiosus*



(tetraethers) (Lobasso et al., 2012) in the total lipid extract of the marine hyperthermophilic archaeon *Pyrococcus furiosus*, which has an optimal cultivation temperature of 100 °C. The total lipid extract was separated by HPTLC: 9-aminoacridine was applied along the entire plate, and negative ion spectra were obtained by means of MALDI-scanning of each spot.

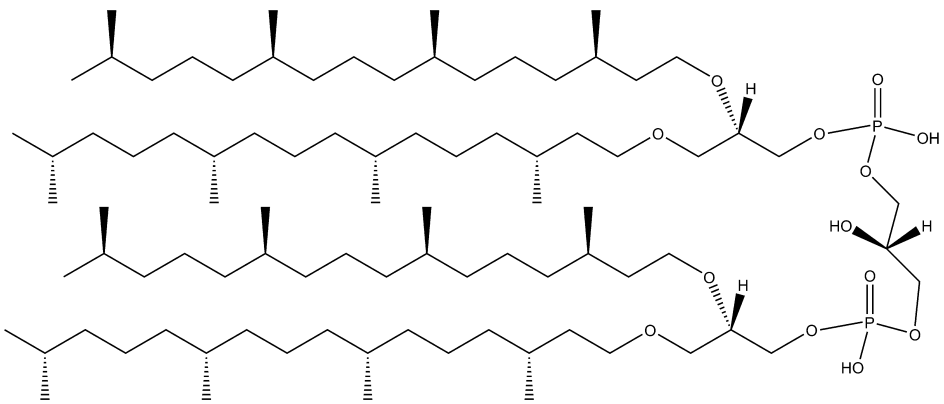
In another study (Angelini, Corral, Lopalco, Ventosa, & Corcelli, 2012), the lipidomes of two extremely haloalkaliphilic archaea, *Natronococcus occultus* and *Natronococcus amylolyticus*, were examined by means of combined TLC and MALDI-TOF/MS analyses. Typical archaeal lipids were again identified, the major being the ether lipid phosphatidylglycerol (PtdGro) and the phosphatidylglycerophosphate (PtdGroP) methyl ester. Furthermore, minor, but hitherto undescribed, lipids of the cardiolipin type were also identified (see Fig. 3), which contained four isoprenoid chains. Based on the analysis, a hypothesis was proposed about the impact of the role of isoprenoid chains on the adaptation of the organism to high pH and high salinity.

A further study (Gagen, Yoshinaga, Garcia Prado, Hinrichs, & Thomm, 2016) described the change in the lipid level in *Thermococcus kodakarensis* during cultivation and especially the increasing content of membrane lipids with tetraether moieties. Chromatographic separation using UHPLC on an amide column coupled with tandem ESI-MS was used to determine the core and polar lipids of *T. kodakarensis* using the internal standard 1,2-dihenarachidoyl-*sn*-glycero-3-phosphocholine. It was found that a putative carbon–nitrogen hydrolase or a membrane-bound hydrogenase complex subunit may affect the production of tetraether lipids.

Analysis of intact membranes from the extremely halophilic archaeon *Halobacterium salinarum* by MALDI-TOF/MS without prior extraction/separation steps has been described by Angelini, Babudri, Lobasso, and Corcelli (2010). 9-Aminoacridine was used as a matrix for the analysis of the whole membrane, and the presence was revealed of many phospho- and glycolipids, for example, bis-PtdGro, glycocardiolipin, PtdGro, phosphatidylglycero methyl phosphate ester, and phosphatidylglycero sulfate, but also complex glycolipids of the types (3′-sulfo)Galp β1-6Manpα1-2Glcpα1–1-[*sn*-2,3-di-*O*-phytanylglycerol] or (3′-sulfo)Galpβ1-6Manpα1-2Glcpα1–1-[sn2,3-di-*O*-phytanylglycerol]-6-[phospho-*sn*-2,3-di-*O*-phytanylglycerol]. The method allowed the identification of the lipids up to a molecular weight of 2000 Da.

TLC in combination with MALDI-TOF/MS identified the structures of glycolipids including a glycosyl-cardiolipin in two halophilic extremophilic microorganisms *Halorubrum trapanicum* and *Haloferax volcanii* isolated from the salt lake near Malaga (Spain) (Lobasso, Perez-

Fig. 3 Structure of an ether cardiolipin (archaeal cardiolipin analog)



BPG (dimeric phospholipid)

Davo, Vitale, Sanchez, & Corcelli, 2015). Besides the "normal" PtdGroP methyl ester, also phosphatidylglycerosulfate or bis-PtdGro were identified as unusual sulfated bis-diglycosyl diphytanylglyceroldiethers with molecular weights of over 1000 Da, and their presence was discussed in relation to high extracellular salinity.

The well-known and frequently studied archaeon *Sulfolobus islandicus* was analyzed by the shotgun approach and was also found to contain, apart from commonly found lipids typical of archaea (dialkyl glycerol diethers and glycerol dialkyl glycerol tetraethers substituted by, e.g. phosphate, inositol phosphate, glycerol phosphate, phospho-glycerol-phosphate or ethanolamine phosphate with one to four hexoses), also sulfonated tri-hexosyl-glycerol dialkyl glycerol tetraether-inositol phosphate (Jensen, Brandl, Treusch, & Ejsing, 2015). Another similar study carried out by shotgun analysis focused on the effects of rising temperatures at which archaea grow an increasing number of cyclopentane moieties in the membranes of *S. islandicus* and *Sulfolobus tokodaii* (Jensen, Neesgaard, et al., 2015).

Elling et al. (2014) dealt with the effects of the growth phase on the membrane lipid composition of the thaumarchaeon *Nitrosopumilus maritimus* and their implications for archaeal lipid distributions by LC–MS in the marine environment. Characteristic glycerol dibiphytanyl glycerol tetraether membrane lipids (GDGT) of marine ammonia-oxidizing archaea were identified by RP-HPLC connected with quadrupole time-of-flight tandem mass spectrometry (QTOF/MS) equipped with an ESI ion source operating in positive mode. TEX86, an organic paleothermometer based upon the membrane lipids of the mesophilic marine *Thaumarchaeota* (Kim, Schouten, Hopmans, Donner, & Sinninghe Damste, 2008), was found to depend on lipidic biomarkers, particularly hexose-phosphohexose GDGT. The authors discussed the biosynthesis, including cyclization to the cyclopentane ring(s) and biodegradation, affecting the reconstruction of the paleotemperature.

The foregoing examples are a mere fraction of published works and should enable the reader to get familiar with the field of analysis of archaea and their unusual lipids. It should be noted that the sample collection, cultivation, and analysis face many problems, which include difficult collection, nonculturability of most archaea (Blum, 2008), or contamination by other microorganisms. All these problems can be gradually solved, but the solution is beyond the scope of this work.

Bacteria

Bacteria constitute a domain of unicellular prokaryotic organisms. They tend to have a coccoid or rod-like shape and usually reach the size of several micrometers (Young, 2006). A typical component of their cell wall is peptidoglycan. The bacterial wall is composed of several layers; the cytoplasmic membrane is a necessary component of all bacteria and consists of a phospholipid bilayer containing transmembrane proteins and glycoproteins. It does not contain sterols (van Heijenoort, 1994).

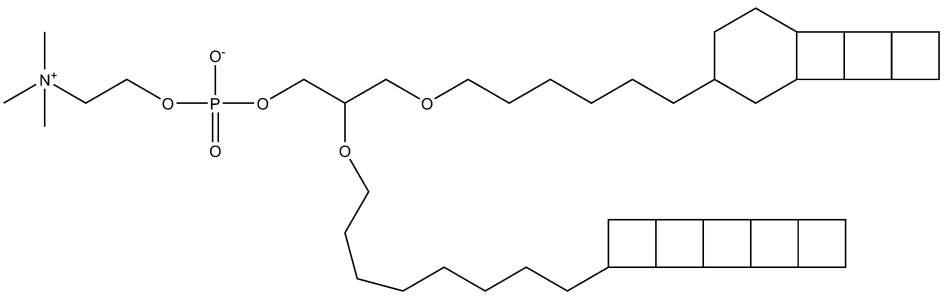
Bacteria contain a huge number of various classes of lipids (Table S1, Supporting information), which fully agrees with the finding that they are among the most widespread groups of organisms in the world.

The lipidome of a single cell can contain up to 100,000 molecular species of lipids (Breslow et al., 2008; Schuldiner et al., 2005; Yetukuri, Ekroos, Vidal-Puig, & Oresic, 2008).

Bacteria possess a wide range of lipid classes, including polyunsaturated fatty acids which have been previously thought to be present exclusively in eukaryotes (Russell & Nichols, 1999). Another very curious group of lipids includes lipids based on cyclobutane ring(s), for example, ladderane phospholipids in anammox bacteria, see Fig. 4 (Lanekoff & Karlsson, 2010).

A comprehensive review of bacterial lipids, metabolism, and membrane homeostasis has been published by Parsons and Rock (2013) and another review describing bacterial membrane lipids and their diversity in structures and pathways by Sohlenkamp and Geiger (2016).

Fig. 4 Structure of ladderane phospholipid in anammox bacteria



Lipidomic analysis of bacteria is a widely used method that has been summarized, for example, in the volume *Introduction to Lipidomics* (Leray, 2012) and in a review (Rezanka, Kresinova, Kolouchova, & Sigler, 2012).

One of the first publications (Gidden, Denson, Liyanage, Ivey, & Lay, 2009) dedicated to lipidomic analysis of bacteria analyzed *Escherichia coli* and *Bacillus subtilis* as two typical representatives of Gram-negative and Gram-positive bacteria, respectively. The published mass spectra of the two bacteria were found to differ vastly. Lipids from both bacteria were analyzed by MALDI-TOF/MS and TOF/TOF tandem MS. *B. subtilis* was found to feature, for example, lysyl-PtdGro and diglucosyl diglycerides, which are missing in *E. coli* that has a different membrane structure. In contrast, *E. coli* was found to contain a unique cyclopropane C17 fatty acid.

Positive and negative desorption electrospray ionization (DESI) MS was used to measure lipids directly, that is, without lipid extraction, in bacterial samples, two Gram-positive bacteria—*B. subtilis* and *Staphylococcus aureus*—and 14 Gram-negative bacteria—*E. coli* and 13 species of *Salmonella.* The data were verified by ESI-MS up to 1000 Da, and the differences between DESI-MS and ESI-MS were found to be only slight. Lipids were further identified using tandem MS. Subsequent principal component analysis (PCA) showed that the species are easily distinguishable from each other and that a different growth medium does not affect the lipid content (Zhang et al., 2011).

S. aureus is one of the best known human pathogens, and has therefore been given great attention. Hewelt-Belka et al. (2014) performed lipid analysis using HPLC-QTOF/MS and created a database of potential molecular species containing 18 main lipid classes, 36 subclasses, and over 7000 lipid molecular species. Much attention has been focused on lipids containing odd-chain fatty acids that occur less frequently than even-chain ones. The possibility was pointed out of comparing strains with different phenotypic characteristics and therefore with different sensitivities to antibiotics.

Cardiolipin (Ptd_2Gro) is a phospholipid comprising four acyl chains and is found in most organisms, especially Gram-negative bacteria such as *E. coli.* Owing to the presence of four acyl chains, it contains a large number of potential molecular species. Analysis by normal-phase liquid chromatography/electrospray ionization mass spectrometry (NP-LC/ESI-MS) of *E. coli* grown at different temperatures resulted in the identification of tens of molecular species, which change depending on the cultivation temperature (Garrett, O'Neill, & Hopson, 2012).

Bacterial cells in a biofilm are physiologically distinct from their planktonic counterparts. RP-LC/ESI-MS was used to measure the composition of the inner and outer membranes of *Pseudomonas aeruginosa* during biofilm formation, and changes of the lipidomic profile of the inner and outer membrane were found to vary with the age of the cells (Benamara et al., 2014).

Phospholipids of the pathogenic strain of *P. fluorescens* were visualized and identified by HPTLC MALDI-TOF/MS. A simple, rapid, and efficient method was described for the screening of bacterial phospholipid classes. This study was the first to identify in *P. fluorescens* phosphatidylcholine (PtdCho), which was till then regarded as a typical eukaryotic phospholipid (Kondakova et al., 2015).

The metabolic profiles of common bacteria (*B. subtilis*, *Streptomyces coelicolor*, *Mycobacterium smegmatis*, and *P. aeruginosa*) were assessed directly using nanospray DESI-MS, that is, without the extraction of lipids from the colonies on Petri dishes. This led to understanding the spatiotemporal dynamics of metabolite production in live microbial colonies (Watrous et al., 2012).

Rapid identification of spores of bacilli (*B. subtilis, B. cereus, B. megaterium, B. pumilus*, and *B. sphaericus*) by MALDI-TOF/MS using lipidomic profiling allowed the identification of phosphatidylethanolamine (PtdEtn), PtdGro, and digalactosyldiacylglycerol (DGDG), which led to their rapid identification under different experimental conditions, for example, when using different matrices (2,3-dihydroxybenzoic acid, sinapinic acid, and 2,4,6-trihydroxyacetophenone) and concentrations. The optimal bacterial concentration for MALDI-TOF/MS was 10^7 cells/mL (Shu et al., 2012).

Among the practical uses of lipidomic analysis by LC–MS is its application in the brewing industry for identification of beer contamination by anaerobic bacteria of the genera *Megasphaera* and *Pectinatus* (Rezanka, Matoulkova, Benada, & Sigler, 2015).

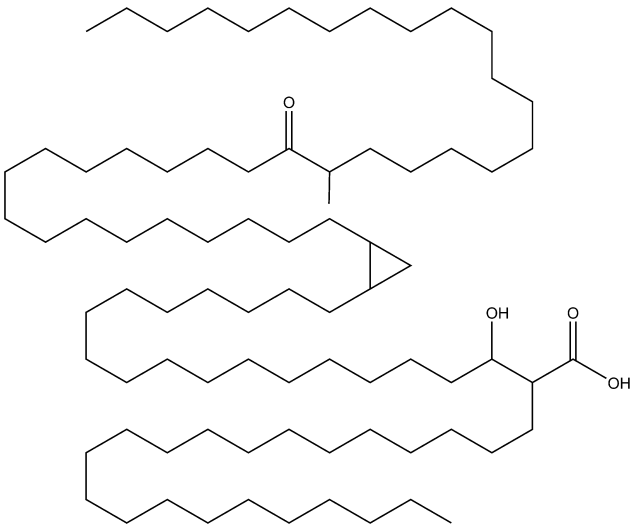
The thermophilic genus *Anoxybacillus* was analyzed by LC/ESI-MS/MS. Analysis of total lipids from the facultatively anaerobic *A. bogrovensis* on a HILIC column succeeded in separating diacyl- and plasmalogen phospholipids. The LC/ESI-MS/MS analysis of the strict aerobe *A. rupiensis* revealed the presence of different unique polar lipids, predominantly alanyl-, lysyl-, and glucosyl-phosphatidylglycerols and Ptd_2Gro (Rezanka, Kambourova, Derekova, Kolouchova, & Sigler, 2012).

Identification of bacterial lipids, particularly in the genus *Clostridium*, which is an anaerobic bacterium, by LC–MS was described by Goldfine and Guan (2017).

Changes determined by shotgun analysis in the lipidomic profile of membrane plasmalogens in *C. pasteurianum* during butanol fermentation were published by Kolek, Patakova, Melzoch, Sigler, and Rezanka (2015).

In terms of the division of bacteria to Gram-positive (G+) and Gram-negative (G−) genera, the published results of lipidomic analysis are rather ambiguous. For instance,

Fig. 5 Structure of a keto mycolic acid in mycobacteria



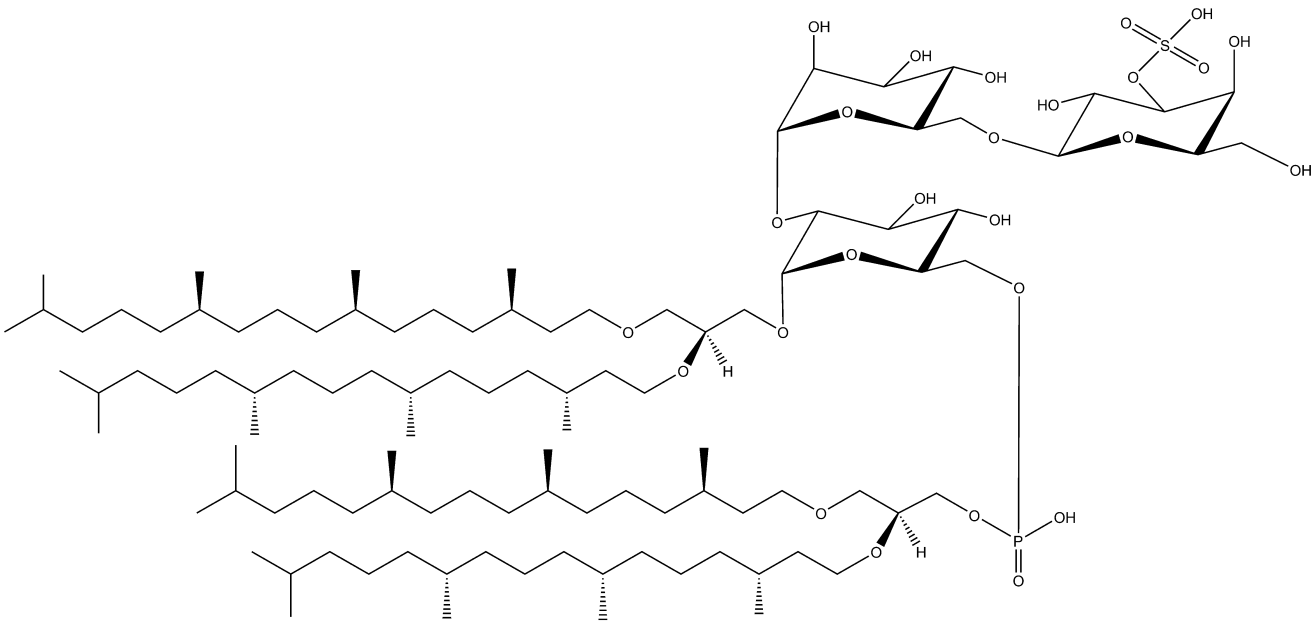
Wei et al. (2016) considered the dividing line between G+ and G− bacteria to be based on PCA analysis, but they did not analyze the typical G− representative *E. coli.* Zhang et al. (2011), while claiming that "The gram-positive and gram-negative bacteria were clearly distinguished using PCA," showed quite clearly in their Fig. 6a, b that, in the case of a positive ionization, *E. coli*, as a representative of G− bacteria, was more related to *S. aureus* (G+ bacterium) than to *B. subtilis* (G− bacterium). An opposite situation was obtained in the case of negative ionization. We therefore believe that, in order for lipidomic analysis to be universally applicable like Gram staining, hundreds of bacteria, ideally those whose entire genome is known, should be analyzed in order to compare the results of lipidomic analysis and genetic analysis.

Actinobacteria (Actinomycetes)

Mycobacteria belong to a genus of nonmotile and nonsporulating bacteria from a separate family *Mycobacteriaceae* belonging to *Actinobacteria.* They are usually considered Gram-positive. The prefix *Myco* refers to the fact that they were once taken as fungi. The genus includes about 100 species, many of them medically significant (Trujillo, 2001). These include some important pathogens, such as *M. tuberculosis* (causative agent of tuberculosis) and *M. leprae* (causative agent of leprosy) and also the *M. avium* complex, *M. kansasii*, *M. abscessus,* and others (Killick et al., 2013). Unlike bacteria, however, they have a complex cell wall, which is hydrophobic, waxy, and rich in mycolic acids/mycolates (Jarlier & Nikaido, 1994) (complex lipids, see Fig. 5).

The lipidomic analysis of mycobacteria gives rise to specific problems because their lipid repertoires range from highly nonpolar polyketides such as phthiocerol dimycocerosates to highly polar phosphatidyl inositol mannosides (see Fig. 6). This has been confirmed by LC–MS identification of more than 30 classes of mycobacterial lipids with more than 5000 molecular species (Layre et al., 2011). Three databases were therefore created in 2011, which focus specifically on genus-specific mycobacterial lipids

Fig. 6 Structure of diacylglycerophosphoinositolmonomannoside lipid in mycobacteria



and were published under the names MTB LipidDB, MycoMass, and MycoMap (Layre et al., 2011; Madigan et al., 2012; Sartain, Dick, Rithner, Crick, & Belisle, 2011).

The complex cell wall of mycobacteria is extremely rich in lipids that constitute about 60% of dry weight. As identified by shotgun analysis, these lipids are arranged in a plasma membrane that consists mainly of anionic phospholipids. The most common fatty acids in the plasma membrane are palmitic acid (PAM), OLA, and tuberculostearic acid. Mycobacteria have not yet been found to contain sphingolipids. Unlike common phospholipids such as PtdGro, Ptd_2GroCL, and PtdEtn, PtdCho has not been found in mycobacteria either. Mycobacteria also produce phosphatidylinositol (PtdIns) and PtdIns mannosides, which exhibit different levels of mannosylation and acylation (Hsu, Turk, Owens, Rhoades, & Russell, 2007a, 2007b).

A separate group of lipids includes mycolic acids, which are not found in other organisms and are composed of α- and β-hydroxy meromycolic chains differing in the length and branching of the chain depending on the type of mycobacteria (Guenin-Mace, Simeone, & Demangel, 2009). The difference in chain length, unsaturation, and substitution of polar groups (—OH, =O, etc.) obtained by using LC–MS can thus provide information useful in chemotaxonomy (Butler & Guthertz, 2001).

A number of lipidomic studies of mycobacteria concerned, for instance, the action of isoniazid on the cells, the use of mechanisms of biosynthesis of mycobacterial lipids as biomarkers of tuberculosis, elucidation of the structures of membranes, and enzyme kinetics (Layre, Al-Mubarak, Belisle, & Moody, 2014).

In conclusion, the lipidomic analysis of bacteria including mycobacteria and their relatives, for example, *Nocardia*, revealed an enormous variability of complex lipids which is not encountered in eukaryotic organisms.

We therefore believe that the nearly explosive development of lipidomic analysis will allow not only chemotaxonomic resolution of bacteria but also the use of lipidomics for clarification of antibiotic resistance and enhancing our knowledge of the behavior of bacteria in biofilms.

Eukaryotic Organisms

A eukaryotic cell (eucellula) is a cell occurring in eukaryotes, which are organisms that have bodies composed of cells with a differentiated nucleus and biomembrane structures. They exist either as separate and single-cell organisms (protozoa, some algae, etc.), or as part of tissues of multicellular organisms (animals, including humans, plants, etc.).

There are many differences between eukaryotic and prokaryotic cells. Cells of eukaryotes are, on average, 10 times larger than the cells of prokaryotic organisms and differ from them in the structure of the nucleus and nuclear chromosomes. They also contain a large amount of organelles enclosed in biomembranes. We distinguish eukaryotic cells of fungi, plants, and animals.

A cell membrane surrounds the entire contents of a live cell. It is similar to the other membranes in the cell and is selectively permeable (freely permeable to uncharged molecules of less than about 150 Da). The basis of biomembranes is a double layer of phospholipids. A cytoplasmic membrane has a hydrophilic (outside) and a hydrophobic (inside) part and contains irregularly spaced protein molecules.

The lipidome of eukaryotic cells consists of hundreds to thousands of molecular species of lipids which form the membrane. Metabolic energy is stored in them, and they also have a function as biologically active molecules (van Meer, 2005; Wenk, 2005; Yetukuri et al., 2008).

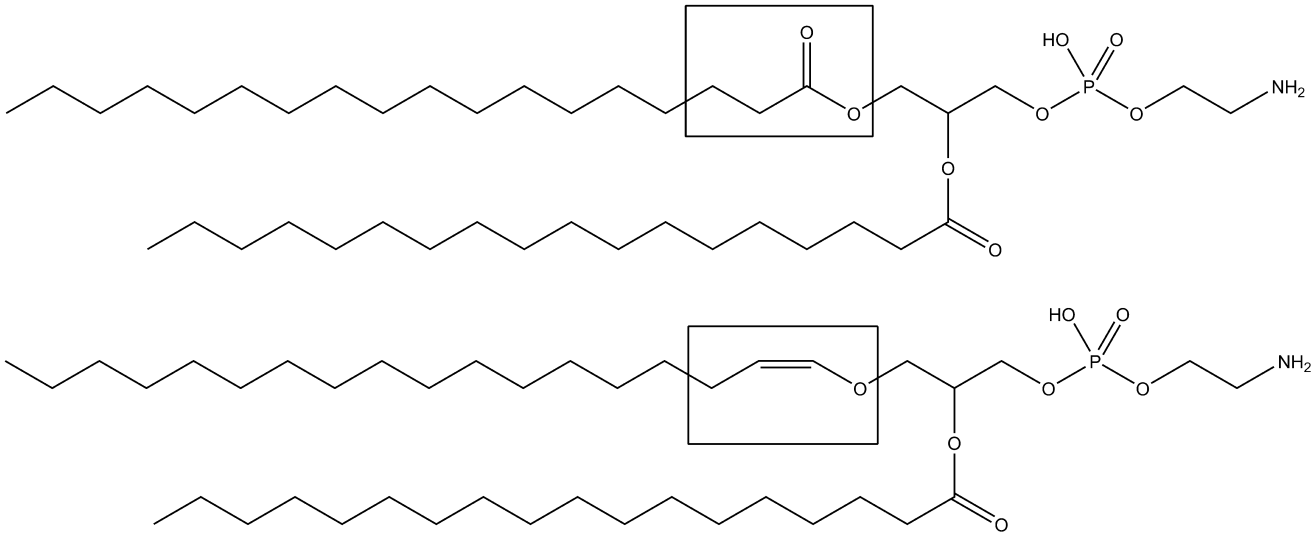
An interesting class of lipids in eukaryotes is constituted by plasmalogens, which are glycerol derivatives containing at the same time the *O*-alkyl moiety at the *sn*-1 position and a fatty acid at the *sn*-2 position. The *O*-alk-1-enyl chain is bound as a vinyl ether, while the fatty acid is bound by an ester linkage, see Fig. 7. The vinyl ether usually has 16 or 18 carbons, the acid being always characteristic for a given group of organisms, for example, saturated and branched (iso or anteiso) acids for bacteria (Rezanka et al., 2012) or polyunsaturated acids for mammals. Phosphoric acid is typically bound at the *sn*-3 position of the glycerol backbone, so that normal plasmalogen types include phosphatidylserine (PtdSer), PtdEtn, PtdCho, PtdGro, or Ptd_2Gro.

Far more interesting than their structure is their narrow distribution in nature, since they are found only in anaerobic bacteria and in animals.

They have not yet been found in aerobic bacteria and plants, including algae (Felde & Spiteller, 1994), and their presence in fungi is still highly questionable (Horrocks & Sharma, 1982).

By contrast, a widespread distribution of plasmalogens in invertebrates (Sugiura, Fukuda, Miyamoto, & Waku, 1992), especially marine sponges (Smith & Djerassi, 1987), and vertebrates (Braverman & Moser, 2012) has been described many times. In mammals, they are particularly widespread in internal organs and brain, heart, kidney, lungs, and skeletal muscle but also in milk (Moukarzel, Dyer, Keller, Elango, & Innis, 2016). They have also been found in clams (Soudant, Marty, Moal, Masski, & Samain, 1998), in insects such as silkworms (Aboshi, Nishida, & Mori, 2012), and in freshwater sponges (Dembitsky, Rezanka, & Kashin, 1994). Their occurrence in invertebrates has been discussed in a review by Magnusson and Haraldsson (2011), who described their representation, both qualitative and quantitative, in dozens of organisms.

Fig. 7 Difference in the structures of diacyl and alkenylacyl ethanolamine



In recent years, plasmalogen analysis has been performed almost exclusively by LC–MS; other methods are either time consuming or inaccurate and usually do not provide information about native plasmalogens and their molecular species. Analysis of plasmalogens by the shotgun method is generally not problematic; see, for example, the study by Hsu et al. (2003), which describes very well their analysis and provides excellent starting information. In our previous study, Rezanka, Matoulkova, Kyselova, and Sigler (2013) on anaerobic bacteria, we separated and identified without problems tens of molecular species. Figure 8 shows a HILIC/APCI-MS chromatogram of the phospholipids from *Pectinatus frisingensis* analyzed by a linear gradient on a semipreparative normal-phase HILIC column.

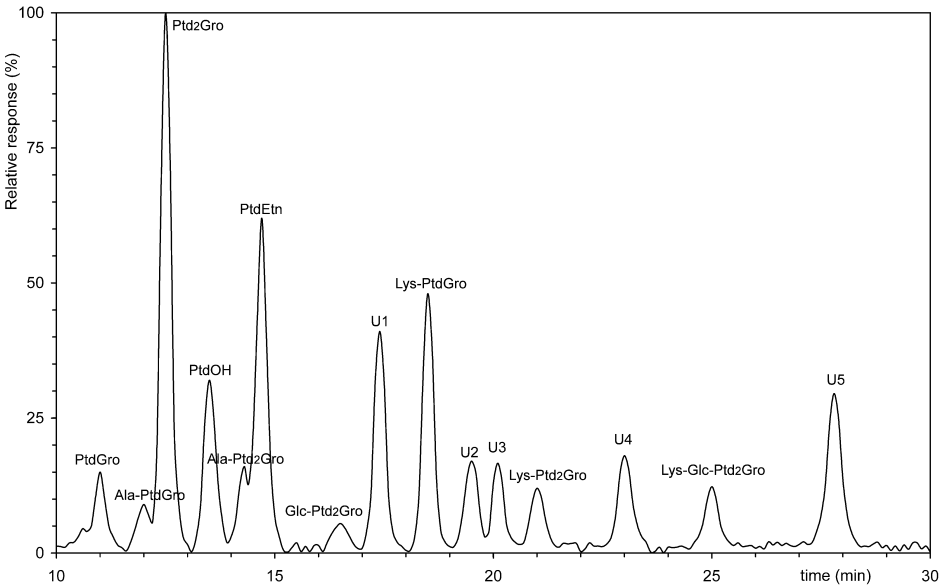
Ether analogs of triacylglycerols, that is, 1-alkyldiacyl-*sn*-glycerols, are present in trace amounts or are missing in most animal tissues, but they can be major components of some marine lipids, for example, those of marine invertebrates. The alkyl moieties usually have a wider range of chain lengths than in other animal tissues. In terrestrial mammals, small amounts of 1-alkyldiacyl-sn-glycerols have been found in liver, adipose tissue, and tumors. In addition to diacyl forms, the membrane phospholipids of many animal and microbial species contain high proportions of molecular species with ether and vinyl ether bonds in position *sn*-1. In annamox bacteria, which contain ladderane fatty acids, the alkyl moieties are in position *sn*-2 of their phospholipids. In animal tissues, the highest proportion of the plasmalogen form is usually in phosphatidylethanolamine, less in PtdCho, and in minor amounts or is absent in PtdIns. The *O*-alkyl form is more abundant than the *O*-alkenyl form, the opposite ratio being found in heart lipids (Magnusson & Haraldsson, 2011; Mangold & Paltauf, 1983; Schmid & Takahashi, 1968).

Yeast

Yeasts are single-celled fungal microorganisms. Most yeasts belong to the class of ascomycetes, some others to the class of basidiomycetes, and they therefore do not form a common taxonomic group. They do not form fruiting bodies, reproduce mostly asexually, and divide usually by budding or fission. They can also reproduce sexually by forming asci, which are not enclosed in any fruiting bodies (ascocarps). They do not form true mycelial structures but only pseudomycelia that resemble colonies of single-celled organisms. Yeasts are widely used in the food industry and biotechnology. Their ability to carry out fermentation is used, for example, in the production of wine, beer, and bread. Some of them are pathogens, for instance *Candida albicans.*

One of the first studies describing the lipidomic profile of the yeast *Saccharomyces cerevisiae* was published more than 10 years ago. Shotgun analysis was used to identify the lipids from 2 million cells, and more than 250 molecular species of lipids belonging to 21 main classes were quantified. The authors assumed that they were able to identify about 95% of the lipidome. They observed a higher proportion of PAM and OLA acids and a lower proportion of palmitoleic acid when the temperature was changed from 24 to 37 °C. Similar changes were observed in phospholipids, which involved an increase in the contents of PtdCho and PtdIns and a drop in the proportion of PtdEtn (Ejsing et al., 2009).

Fig. 8 Hydrophilic interaction chromatography/atmospheric pressure chemical ionization-mass spectrometry (HILIC/APCI-MS) chromatogram of the phospholipids from *Pectinatus frisingensis*, analyzed by a linear-gradient semipreparative, normal-phase high-performance liquid chromatography (HPLC)-HILIC column. Ux is unknown



Comparative lipidomics of parental strains and a recombinant xylose fermenting yeast strain of *S. cerevisiae* performed by UHPLC–MS/MS identified over 100 molecular species of lipids (Xia et al., 2011) in order to clarify the utilization of xylose for fermentation. Differences were found in PtdIns levels in relation to the metabolism of xylose and in the PtdIns/PtdSer ratio depending on the growth capacity.

Shotgun lipidomic analysis of *S. cerevisiae* was performed during cultivation (Casanovas et al., 2015). The presence of 21 lipid classes versus time was monitored. This showed dramatic changes in the representation of both lipid classes (e.g. PtdEtn or PtdIns) as well as changes in the concentration of molecular species. Striking is the change in, for example, PtdCho34:2 versus PtdCho32:2 or PtdOH34:2 versus PtdOH32:2.

Shotgun lipidomic analysis of cells cultivated on acetic acid was performed in *S. cerevisiae* and *Zygosaccharomyces bailii.* Acetic acid in its undissociated form penetrates into cells and changes their phospholipid content including an increase in PtdIns or a rise of all classes of sphingolipids: inositolphosphoceramide (InsPCer), mannosyl-inositolphosphoceramide (MInsPCer), and mannosyl-diinositolphosphoceramide ($M(InsP)_2Cer$). As a result, the tolerance to acetic acid was found to lead to an increase in the content of sphingolipids, especially in *Z. bailii* (Lindberg, Santos, Riezman, Olsson, & Bettiga, 2013).

Hundreds of lipid species were analyzed by the shotgun method. They included glycerophospholipids, sphingolipids, and sterols, which were quantified in a collection of 129 mutants of *S. cerevisiae* differing in protein kinase and phosphatase genes (Silveira Dos et al., 2014). The authors analyzed yeast with deletion of nonessential genes encoding kinases or phosphatases and investigated the effect on lipid composition. The wild type and its mutants were found to differ, for example, in the content of molecular species of PtdCho (32:2 or 34:2 PtdCho).

A similar work, but with the absence of genes YBR141C and YJR015W with unknown function, was performed by shotgun analysis by a similar group of authors, again in *S. cerevisiae* mutants (Tarasov et al., 2014).

Another study reported on lipidomic profiling of *S. cerevisiae* and four other hemiascomycetous yeasts, that is, *S. bayanus, Kluyveromyces thermotolerans, Pichia angusta*, and *Yarrowia lipolytica* (Hein & Hayen, 2012). Lipidomic analysis by LC–MS revealed more than 100 molecular species belonging to nine phospholipid classes. The major lipids were Ptd_2Gro, PtdEtn, PtdCho, PtdIns, PtdSer, and PtdGro. The most common molecular species were those containing OLA and palmitoleic acids (molecular species 34:2). The characteristic profile was detected of each yeast, two species of the genus *Saccharomyces* being the most closely related.

A study of the effect of the culture temperature (15, 24, 30, and 37 °C) on the lipidomic profile of wild-type *S. cerevisiae* and its mutants has been published by Klose et al. (2012). The results showed that increasing temperature causes an increase in the content of PtdIns and decrease of the content of TAG and PtdEtn. The most

striking differences were found in molecular species with the sum of 44 carbon atoms, zero double bond(s), and 4 hydroxyl groups in the long chain base (i.e. InsPCer 44:0;4) or 32:2 PtdCho.

Lipidomic analysis by LC–MS was carried out on the methylotrophic yeast *Pichia pastoris* (Klug et al., 2014), and the profiles of three lipidomic fractions were compared, that is, the homogenate, 30,000*g*, and 40,000*g* microsomes. The most abundant were 54:3 TAG, 36:4 PtdCho, and 34:1 PtdIns. Unfortunately, the authors used tandem MS for analysis of only sphingolipids but not TAG or phospholipids. The result is, for example, the discovery that TAG 54:3 is a combination of eight molecular species, phospholipid C34:2 of four molecular species, and so on. This example clearly shows that the failure to utilize the possibilities that the mass spectrometer offers results in a loss of information and dramatically lowers the value of the overall results.

From the above examples, it follows that the findings in the field of yeast lipidomics are very scarce and experiments are basically confined to *S. cerevisiae* as one of the best culturable yeasts. Further developments in this area can be seen primarily in lipidomic analysis of less common and less easily culturable yeasts such as psychrophilic yeast (Rezanka, Kolouchova, & Sigler, 2016).

To our current knowledge, thorough lipidomic analysis of fungi except for yeast has not yet been performed. Basically, only some molds have been analyzed, but usually only for a specific reason, not as a whole lipidome as has been done in yeast. An instance is the study of the symbiosis between *Rhizopus microsporus* and *Burkholderia* (Lastovetsky et al., 2016) concerned with the effects of diacylglycerol kinase on the relative abundance of phospholipid and neutral lipid species in cured host *R. microsporus* grown with and without *Burkholderia.*

In another publication (Xu et al., 2012), the authors used lipidomic LC–ESI-MS^n analysis for investigating the effects of changes in fermentation conditions in *Cephalosporium acremonium* in industrial and pilot fermentations. A total of 77 phospholipid species were detected, and 65 species were further quantified. Major differences were found between PtdIns, PtdSer, and PtdOH in the industrial and pilot processes. The identified common molecular species included, for example, 18:1/18:1-PtdOH, 18:3/18:3-PtdOH, 16:0/18:3-PtdSer, 18:2/18:3-PtdSer, 16:0/18:3-PtdIns, and 18:2/18:3-PtdIns. As the authors demonstrated using multivariate statistical analysis, differences in the representation of the markers between the industrial and the pilot process in dependence on the cultivation length were absolutely significant.

The lipidomic profile of *Penicillium chrysogenum* was determined by LC–ESI-MS^n (Qiao, Lu, Cao, Chen, & Yuan, 2013) in industrial-scale fermentations. During the fermentation, cells were shown to absorb significant amounts of exogenous saturated FA and polyunsaturated fatty acids (PUFA are FA with methylene-interrupted double bonds, that is, with two or more double bonds of the cis-configuration separated by a single methylene group) from the feedstock, and incorporate them into their cell membranes. Large differences were noted between pilot and industrial fermentations in phospholipid molecular species containing PUFA, which dramatically influence cell membrane fluidity. This phenomenon has been used to optimize industrial-scale fermentation.

Cell wall lipids from *Paracoccidioides brasiliensis* grown in the presence and absence of human plasma (Longo et al., 2013) have been characterized. Using electrospray ionization and gas chromatography-mass spectrometry analyses of lipids extracted from different isolates, the authors identified phospholipids, fatty acids, sterols, neutral glycolipids, and phospholipids including PtdCho, PtdEtn, PtdSer, PtdGro, PtdIns, and PtdOH. In addition to common molecular species, less common ones, for example, 19:1/19:2-PtdCho, 16:0/19:1-PtdOH, 16:0/20:2-PtdSer, 16:0/19:2-PtdIns, have been found. The study aided in understanding their role in fungal biology as well as interaction with antifungal drugs and with the host. Once again, the most common molecular species of phospholipids were 18:3/18:3-PtdCho, 18:3/18:2-PtdCho, 16:2/18:2-PtdCho, or 16:2/18:1-PtdCho.

In conclusion, lipidomic analysis of fungi, in particular molds, is only at the beginning of its development, and a rapid increase in publications can be expected, particularly concerning species having industrial use such as food production (the citric acid producer *Aspergillus*), the manufacture of pharmaceuticals from molds, or production of mycotoxins.

Cyanobacteria and Algae

Cyanobacteria

Cyanobacteria are substantially Gram-negative bacteria, except for their lipid content, which is much like that of eukaryotic algae, see below. They are characterized by their ability to perform photosynthesis, which generates oxygen. The cells of cyanobacteria are unicellular or filamentous, mostly blue-green-colored (see the earlier name blue-green algae), and in many respects are typical prokaryotes (Singh, Sinha, & Hader, 2002). The main pigments involved in photosynthesis include chlorophyll (usually type a, sometimes b, c, or d) and others (Vothknecht & Westhoff, 2001). Cyanobacteria reproduce asexually by cell division or fragmentation of filaments. They occur very frequently

in the aquatic environment, but also in soil and often in extreme conditions, such as deserts or arctic areas. They enter very often into symbiotic relationships. In addition to endosymbiotically incurred plastids, there are many cases where cyanobacteria help their hosts in nitrogen or carbon fixation (Castenholz et al., 2001).

Cyanobacteria contain glycolipids such as monogalactosyldiacylglycerol (MGDG), DGDG, SQDG, and PtdGro as the main representative phospholipids. Cyanobacterial lipids are similar to membrane lipids of chloroplasts of higher plants and are distinct from the membranes of most bacteria, which contain phospholipids as the main glycerolipids (Somerville, 1995).

Algae

Algae are simple photosynthetic organisms, traditionally ranked among lower plants. In fact, algae are a set of unrelated groups of organisms, and only some of them are close to plants. Algae are both unicellular and multicellular, the bodies of multicellular algae being formed by a thallus. Most of them are not able to survive in a dry environment and live therefore in fresh or salt water. Land-dwelling forms are small, inconspicuous, and can be found abundantly in humid tropical areas. Some algae have solved the problem of drying by entering into symbiosis with fungi and forming a constituent part of lichens.

Most algae are capable of photosynthesis and are therefore auxotrophic, but they can be also cultivated heterotrophically. Algae are part of aquatic ecosystems; red algae and seaweed provide food and shelter for aquatic animals, and microscopic algae form phytoplankton. Eukaryotic algae are a diverse group of organisms that contain and produce a variety of lipids. Though the lipid content of algae has been less intensively studied than that of higher plants, there is enough information to point out some general features. Algae contain many major plant-like lipids, for example, glycosylglycerides and common phospholipids. In addition, they contain less common compounds such as betaine lipids or sulfolipids. Like in plants, the main acyl lipids are three glycosylglycerides, that is, MGDG, DGDG, SQDG, as well as PtdGro. These lipids contain characteristic fatty acids, often with a high content of polyunsaturated fatty acids, which may comprise up to six double bonds (Pignolet, Jubeau, Vaca-Garcia, & Michaud, 2013).

We should also mention the "algae" of the dinoflagellate type, which, however, are in fact protists and are called algae only for commercial reasons. They commonly contain 18:4 and 18:5 PUFA; it should be noted that octadecapentaenoic acid is lacking in higher plants. They have been found to contain the as-yet most highly unsaturated PUFA; a typical example is 28:8ω-3 acid. Their lipidome is still almost unexplored (Rezanka, Nedbalova, Prochazkova, & Sigler, 2014).

Lipids of cyanobacteria and algae are largely similar to lipids of higher (flowering) plants, but algae often contain special classes of lipids absent in higher plants. Typical examples are diacylglyceryltrimethylhomoserine (DGTS), diacylglycerylhydroxymethyltrimethylalanine (DGTA), and diacylglycerylcarboxyhydroxymethylcholine (DGCC), see Fig. 9. Unlike bacteria and yeast, algae contain often more than 50% of PUFA with 20 or more carbons, which are also lacking in higher plants. Examples include 20:4, 20:5, and 22:6 acids.

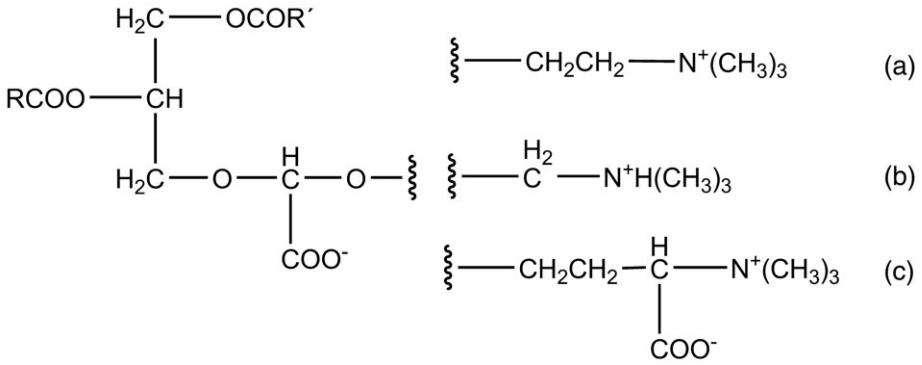
The lipidomic profile of algae is very often assessed primarily to determine how stress conditions during cultivation affect lipid composition. One of the often used stresses is salt stress. It is usually caused by a higher concentration of NaCl in a culture medium in which freshwater algae grow. In a series of works, Lu, Wei, Chen, and Yang (2013) studied the effects of NaCl stress and stress caused by nitrate or phosphate deprivation in the alga *Chlamydomonas nivalis.* Using UHPLC/ESI-MS in positive-ion and negative-ion modes, they identified, apart from common phospholipids, lipid biomarkers such as DGTS, MGDG, DGDG, and SQDG. Some molecular species contained a less common PUFA, that is, 16:4. Multivariate statistical analysis clearly identified lipids obtained under various culture conditions (control, nitrate-, and phosphate-deprived) (Lu et al., 2013).

Further studies focused on salt stress, using again positive and negative LC/ESI tandem MS on the same alga cultured at different NaCl concentrations (0.25, 0.5, and 1%), and reported on a different composition of molecular species of lipids, which was again statistically evaluated (Lu et al., 2013; Lu, Wei, Jiang, Chen, & Yang, 2012).

Another group of studies described the difference in the lipidomic profiles in different varieties of algae. UHPLC-QTOF/MS was used to distinguish morphologically similar microalgae of two different strains of *Nannochloropsis oceanica*, which were indistinguishable by 18S rRNA and morphological features (Li et al., 2015). The use of statistical processing of data (e.g. PCA) obtained by positive and negative ESI-MS revealed differences in the representation of polar lipids such as DGTS, MGDG, DGDG, and SQDG. Some identified molecular species belonged to those that are not commonly encountered: DGTS (20:4–20:5), MGDG (20:5–14:0), DGDG (20:5–16:1), and also, for example, TAG (16:1–20:5–20:5).

The lipidomic profile of lipids from geographic varieties of the snow alga *Chloromonas pichinchae* was investigated using silver-LC/APCI-MS and NARP-LC/APCI-MS (Rezanka et al., 2014), see Fig. 10. The main focus was on the analysis of TAG-containing PUFA. Several tens of TAG were identified and semiquantified, for example, the

Fig. 9 Structures of betaine lipids (A = 1,2-diacylglyceryl-3-*O*-4′-(*N*,*N*,*N*-trimethyl)-homoserine, B = 1,2-diacylglyceryl-3-*O*-2′-(hydroxymethyl)-(*N*,*N*,*N*-trimethyl)-β-alanine, and C = 1,2-diacylglyceryl-3-*O*-carboxy-(hydroxymethyl)-choline)



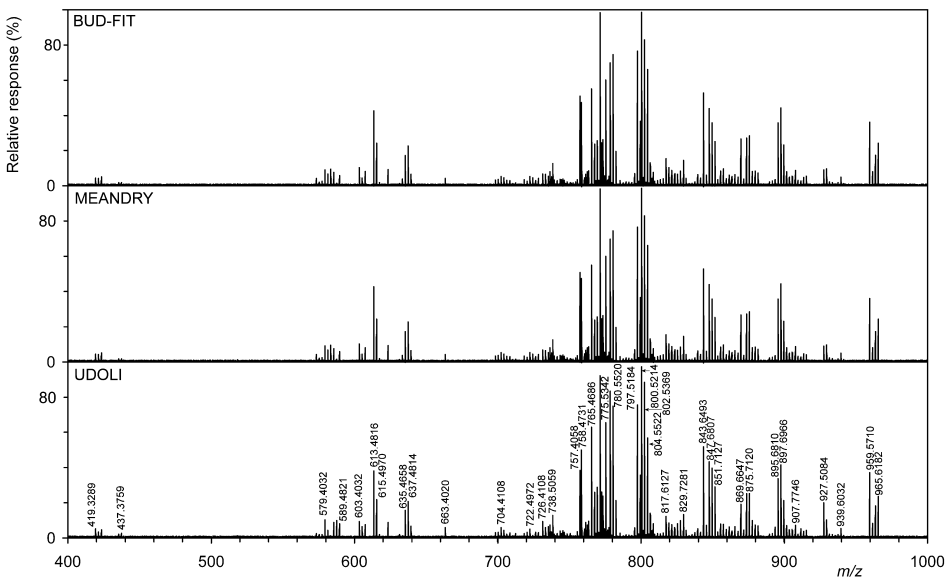
TAG 16:4/16:4/18:4 or 16:3/16:3/18:4. Besides these, TAG were identified in other polar lipids, for example, SQDG 18:4/18:4. The snow alga was thus found to be a potential excellent biotechnological source of PUFA.

Lipidomic analysis was performed on the extremophilic red alga *Galdieria sulphuraria* (Vitova, Goecke, Sigler, & Rezanka, 2016). Analysis of polar lipids performed by HILIC-LC/HR ESI-tandem MS identified 14 classes of lipids including tens of molecular species of lipids, including regioisomers. This alga grows at low pH, and the lipidomic profile was therefore investigated in cells cultivated at pH 1 to 4. The alga was found to utilize various mechanisms for adaptation to low pH, for example, substitution of phospholipids for betaine lipids or a change in the ratio of regioisomers of polar lipids, that is, phospho-, glyco-, and betaine lipids, depending on the pH of the culture medium.

Analysis of polar lipids using a nanoelectrospray direct-infusion MS method was performed on the alga *Chlamydomonas reinhardtii* (Yang et al., 2015). The use of positive and negative mode ESI identified polar lipids common in algae (DGTS, MGDG, DGDG, and SQDG) including their molecular species (DGTS 16:0/18:4, SQDG 16:0/18:3, PtdGro 16:1/18:3, etc.).

Another group of studies describes the production (overproduction) of lipids, mostly as a source of biofuel or TAG. The profile of TAG was analyzed in four oleaginous salt-water microalgae *Phaeodactylum tricornutum, Nannochloropsis salina, N. oculi*, and *Tetraselmis suecica* by MALDI-TOF/MS, ESI linear ion trap-orbitrap MS, and ^{1}H

Fig. 10 Mass spectrum (positive ion mode) of total lipids from the snow alga *Chloromonas pichinchae*, collected from different geographical locations, acquired by high-mass-resolution electrospray ionization-mass spectrometry (ESI-MS). Numbers correspond to accurate mass values



NMR spectroscopy (Danielewicz, Anderson, & Franz, 2011). Dozens of TAG have been identified, but their regioisomers were not determined. Examples are TAG 20:5/20:5/20:4 or 16:1/16:3/20:5 and many others. The method thus provides a fast and accurate analysis useful in the production of biofuels.

The lipidomic profile of algal crude oils from *N. salina* analyzed without LC but using ESI, APCI, APPI, and MALDI showed large differences in the abundances of individual classes of lipids and their molecular species (Lee et al., 2013). This work is a beautiful example of how important it is to use internal standards in the quantification, although it is clear that not all the necessary standards are commercially available.

UHPLC-HR-MS identified more than 100 molecular species of TAG in six algae of the genera *Botryococcus, Nannochloropsis, Neochloris, Phaeodactylum, Porphyridium*, and *Scenedesmus* (MacDougall, McNichol, McGinn, O'Leary, & Melanson, 2011). *B. braunii* was found to contain TAG with the longest chain FA, that is, 28:1/28:2/18:1 or 28:2/28:2/18:1. In addition, *S. obliquus* contained TAG containing 20:5/18:3/16:3 PUFA. This work, performed more than 5 years ago, showed in full the advantages of lipidomic analysis, which makes it possible without any problem to identify and partly also quantify TAG with completely different structures. Two recent papers, one review (Richardson et al., 2017) and a paper (Chen et al., 2017), have described the metabolome of marine algae.

Higher Plants

Higher plants (*Embryophyta*, *Cormobionta*, *Embryobionta*, *Telomophyta*) form a subkingdom of multicellular green plants adapted to life on land. Some species are secondarily aquatic. Typical for their life cycle is alternation of generations, with an alternating sexually reproducing generation (gametophyte) with generations that reproduce asexually (sporophyte). Higher plants are derived from green algae, but their body is not formed by a thallus but is differentiated to various plant organs. The organization of the body is still near the thallus only in the most primitive higher plants. The thylakoids of chloroplasts contain chlorophyll *a* and *b*. Seed plants (*Spermatophyta*) form a group that includes all plants that produce seeds. These plants have been examined for centuries mainly for their content of physiologically active compounds, for example, alkaloids, saponins, and others. They are the main producers of lipids, mainly TAG, which serve as food or have industrial uses (biofuels). They have also been given great attention even in the field of lipidomic analysis. We present here a brief survey of the reviews that have been published on the subject in the last decade.

In their review of the analysis of vegetable lipids, Hummel et al. (2011) were among the first to describe the use of UHPLC/HR-MS. Like many others, they focused on the analysis of 260 molecular species of nonpolar and polar lipids from plant (*Arabidopsis*) leaves. The function of the polar lipids from this plant was explained in the review by Nakamura (2015). This plant belongs to the family *Brassicaceae*, and was the first plant the genome of which has been sequenced. It has therefore become a very popular experimental (laboratory) plant. A total of 15 classes of lipids were identified in it, which include PtdCho, PtdEtn, PtdGro, PtdIns, PtdSer, MGDG, DGDG, SQDG, TAG, DAG, and FA, but also some less common ones such as ceramide (Cer), glucosylceramides (GlcCer), and glycosylinositolphosphoceramides (GlcInsPCer).

A recent review by Michaelson, Napier, Molino, and Faure (2016) is focused on plant sphingolipids, including complex GlcCer, InsPCer, and GlcInsPCer. A special method, namely mass spectrometry imaging (Boughton, Thinagaran, Sarabia, Bacic, & Roessner, 2016), which they used has a huge potential. Like nearly all the groups of organic compounds found in plants, also lipids were analyzed by this method. Here are a few examples of lipidomic analysis performed in *Arabidopsis.* Continuous infusion of polar lipids such as glycerolipids (Devaiah et al., 2006), trigalactosyldiacylglycerols (by LC–MS) (Lu, Xu, Awai, Jones, & Benning, 2007), or nonpolar TAG (direct-infusion) (James et al., 2010) was performed in different *Arabidopsi*s mutants. Furthermore, the use of LC–MS made it possible to determine changes in the lipidomic profile of the *Arabidopsis* glycerolipidome in response to light and temperature (Burgos et al., 2011).

In another publication, Lu, Napier, Clemente, and Cahoon (2011) focused on oilseeds engineered for an enhanced or altered lipid metabolism by shotgun analysis.

Two industrially important plants, that is, tobacco and linseed, have been investigated for the biosynthesis of very-long-chain fatty acids (Abbadi et al., 2004). Another massively cultivated plant, soya, was investigated for the content of the LL-PtdCho and LL-PtdEtn in mutant strains (Lee, Welti, Schapaugh, & Trick, 2011).

In general, lipidomic studies of higher plants were mostly focused both on new methods, for example, localization of lipids in tissues and cells, and the use of lipidomic analysis as a tool for increasing lipid production by plants.

A study of *Camelina sativa* seed lipids (Horn & Chapman, 2014) has shown the possibilities of visualization by MALDI-TOF of individual molecular species of TAG (GAGALA, LNAGALNA) or PtdCho (GALA, GALNA).

Leftovers after pressing oil from the commercially important oil plants (camelina, flaxseed, hemp, sesame, and walnut) were analyzed using a shotgun lipidomics

approach, as well as hyphenated RPLC- and NPLC–MS. PUFA such as ALA and LA were identified in phospholipids (PtdOH, PtdIns, PtdEtn, PtdCho, PtdGro, PtdSer, Ptd_2Gro), and tens of molecular species were determined, with ALA acid being located in the *sn*-2 position of the glycerol backbone (Bure et al., 2016).

Tenenboim, Burgos, Willmitzer, and Brotman (2016) used lipidomic analysis for expanding the knowledge on lipid metabolism in plants. HILIC-MS/MS identified a new lipid, namely glucuronosyldiacylglycerol, whose accumulation functions as a protection mechanism during phosphorus starvation.

Another possibility of using mass spectrometry imaging is the use of lipidomics in tissues, cells, and subcellular compartments (Horn & Chapman, 2012), for instance, for the detection of the heterogeneity of lipid droplet subcellular organelles by direct MS.

Animals

Animals (*Metazoa*, *Animalia*) are a kingdom of multicellular heterotrophic organisms that are different from plants and fungi at the cellular level. Their cells have no plastids or cell wall. As heterotrophic organisms, animals are dependent on autotrophic organisms, particularly on plants. Some animals live in symbiosis with unicellular autotrophic organisms (e.g. sponges or corals with cyanobacteria or algae) which provide them with food. The animal body is characterized by a considerable specialization of its individual parts (tissues, organs). Tissues and organs of animals can then be assigned to two primary germ layers formed during embryogenesis, that is, external ectoderm and internal endoderm. Developmentally advanced bilateria have also an intermediate mesoderm. Lipidomic analysis of animals is still far from systematic. Almost neglected is the huge group of invertebrates, for example, sponges, snails, butterflies, and so on, as well as some vertebrates, for example, amphibians and reptiles, while conventional laboratory animals (mice, rats) have been intensively analyzed. Also human organs are often analyzed for the detection of diseases, either genetically inherited or those arising during their lifetime (diabetes, cancer, Alzheimer's disease, cardiovascular disease (CVD), dementia, etc.

Below are just a few examples of lipidomic analysis. As stated above, in many animal groups, such as sponges, the analysis has not yet been performed. In our opinion, this group is unjustly neglected because sponges are characterized by unusual FA (e.g. demospongic acids, which are very-long-chain fatty acids (VLCFA) that have 22 or more carbons).

The lipidome of fishes has also been studied only infrequently. For example, Kakela et al. (2008) followed the seasonal changes of the brain lipidome in crucian carp (*Carassius carassius*) and found that they are mainly related to the ambient temperature.

The differences in polar lipids of the sera of two groups of fishes (yellow croaker *Pseudosciaena crocea* and Japanese seabass *Lateolabrax japonicus*) were investigated after a tropical storm (Yan et al., 2012).

Shen et al. (2012) analyzed by shotgun lipidomics the phospholipid content of fishery wastes for their further utilization. They identified dozens of molecular species of all the major phospholipid classes (PtdSer, PtdIns, PtdCho, PtdEtn, etc.); the major ones included also the less usual PtdSer 18:0/22:6, PtdIns 18:0/20:4, and PtdIns 18:0/20:5. An advantage of shotgun lipidomics is the more than 10-fold shortening of the time of analysis, which aids in the exploitation of unconventional resources as valuable secondary raw materials.

Studies concerned with lipidomic analysis of the three most frequently examined mammals, that is, mice, rats, and humans, have been published nearly 200 times only in 2016. Therefore, in this section we will focus only on quoting several reviews (Bhattacharya, 2013; Butovich, 2011; Colsch, Seyer, Boudah, & Junot, 2015; Gooley & Chua, 2014; Kolovou, Kolovou, & Mavrogeni, 2015; Touboul & Gaudin, 2014; Yang et al., 2016; Zhao, Cheng, Lin, & Wei, 2015) and on a selection of the most promising original research. Lipidomic analysis is primarily used to detect changes in the lipid profile of the brain (Gaudin et al., 2014; Proitsi et al., 2015; Zhang, Chen, Liang, & Zhang, 2015), kidney (Cifkova et al., 2015), blood (Surma et al., 2015), or liver (Park et al., 2016), but also other tissues and secretions of mammals, for example, horse semen (Wood, Scoggin, Ball, Troedsson, & Squires, 2016). In essence, the lipidomic analyses in humans are most commonly published in relation to various diseases or their prevention.

Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum disease are peroxisome biogenesis disorders. As a result of impaired peroxisome function, an individual's own cells and tissues can accumulate very-long-chain fatty acids and branched-chain fatty acids that are normally degraded in peroxisomes. Abe, Honsho, Nakanishi, Taguchi, and Fujiki (2014)) described the use of lipidomic analysis to identify aberrant metabolites in fibroblasts from patients with Zellweger syndrome, acyl-CoA oxidase 1 deficiency, D-bifunctional protein, and X-linked adrenoleukodystrophy, as well as in peroxisome-deficient Chinese hamster ovary cell mutants. The study revealed the presence of 20:4/28:5, 18:1/30:8, and 22:4/26:5 PtdCho. This pioneering study documents that the use of lipidomic analysis of such molecular species that contain VLCFA and/or VLCPUFA is still in its infancy.

Conclusion

Further development of lipidomic analysis can be expected in two directions. One of them is the application of new ionization techniques, such as atmospheric pressure photoionization mass spectrometry and tandem MS, particularly when associated with the use of high-resolution mass spectrometry by many time-of-flight mass spectrometers, as well as the use of ultrahigh-performance liquid chromatography. This direction gains in importance since the prices of cutting-edge devices have been falling and this instrumentation is increasingly more accessible to a wider range of consumers.

The other direction is in the possibility of analyzing a much more extensive range of samples, especially those from the group of microorganisms, invertebrates, and lower plants, including algae, or associated with special and infrequently occurring diseases, often congenital, in humans. To our knowledge, LC–MS and shotgun analysis have not yet been used for analyzing materials from various bacteria, yeasts, fungi, and/or protozoa or sponges containing very long chain fatty acids or very long chain polyunsaturated fatty acids. As a result, the next decade is very likely to witness explosive development in this field.

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