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Lipidomic analysis of bacterial plasmalogens

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Abstract Plasmalogens are a group of lipids with potentially important, and not yet fully known, functions in organisms from bacteria to protozoans, invertebrates, and mammals. They can protect cells against the damaging effects of reactive oxygen species, protect other phospholipids or lipoprotein particles against oxidative stress, and have been implicated as signaling molecules and modulators of membrane dynamics. They have been found in many anaerobic bacterial species, and their biosynthetic pathways differ in aerobic and anaerobic organisms. The use of advanced techniques permits the identification of not only plasmalogen classes but also their positional isomers and often also individual molecular species. This paper describes direct analyses of plasmalogens from natural sources, frequently very unusual, using electrospray ionization mass spectrometry in combination with high-performance liquid chromatography and/or shotgun lipidomics.

Abbreviations

ACN	Acetonitrile
AcOLi	Lithium acetate
$AcONH_4$	Ammonium acetate
CID	Collision-induced dissociation
CL	Cardiolipin
DHAP	Dihydroxyacetone phosphate
ESI-MS	Electrospray ionization mass spectrometry
EtOH	Ethanol
FAB	Fast atom bombardment
FAs	Fatty acids
GC	Gas chromatography
HILIC	Hydrophilic interaction liquid chromatography
i-PrOH	Propan-2-ol
IT	Ion trap mass spectrometer
LPC	Lysophosphatidylcholine
MALDI	Matrix-assisted laser desorption ionization
MeOH	Methanol
MSMA	Magnetic sector mass-analyzer
Nano-ESI-MS-MS	Nano-electrospray ionization tandem mass spectrometry
ND	Not detected
PC	Phosphatidylcholines
PE	Phosphatidylethanolamines
PG	Phosphatidylglycerols
PLs	Phospholipids
Pls	Plasmalogens
PlsPC	Plasmalogen phosphatidylcholine
PlsPE	Plasmalogen ethanolamines
PlsPG	Plasmalogen phosphatidylglycerol
PlsPS	Plasmalogen phosphatidylserine
PS	Phosphatidylserine
Q	Quadrupole mass spectrometer
QqQ	Triple quadrupole mass spectrometer
QTOF	Hybrid quadrupole time of flight mass spectrometer
ROS	Reactive oxygen species

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TIC	Total ion current
TLC	Thin layer chromatography

Occurrence

Plasmalogens are derivatives of glycerol, which are characterized by the presence of vinyl ether linkage at the *sn*-1 position and an ester linkage at the *sn*-2 position (Fig. 1) (Goldfine 2010). The *sn*-1 position is occupied by fatty alcohol having predominantly 16 and/or 18 carbon atoms but also odd-numbered and unsaturated chains were discovered in bacteria (Nagan and Zoeller 2001; Farooqui and Horrocks 2004; Rezanka et al. 2011). Fatty acids (FAs) are bonded in *sn*-2 position of glycerol; the situation in bacteria is again complicated; common bacterial acids usually present in this position are branched or even-numbered straight chain ones (Rezanka et al. 2011). The most common polar group linked to glycerol in *sn*-3 position is phosphatidylserine (PS), phosphoethanolamine, or phosphatidylglycerol (PG). The plasmalogen form of cardiolipin has also been found.

Plasmalogens have unique distribution in the nature, since they occur only in strictly anaerobic bacteria, but not in aerobic or facultatively anaerobic bacteria (Table 1). They are not present in plants (Feld and Spiteller 1994), and their presence in fungi is very questionable (Horrocks and Sharma 1982). It has not been proven by anyone in the last 20 years, so we believe their finding described by Feld and Spiteller (1994) to be due to an incorrect analysis.

By contrast, plasmalogens are widespread in invertebrates and vertebrates. In mammals, they are particularly widespread in internal organs, such as the brain, heart, kidney, lungs, and skeletal muscle.

Biosynthesis

Biosynthesis of plasmalogens in higher organisms, which has been thoroughly described (see Fig. 2 in Goldfine 2010), consists in principle in acylation of dihydroxyacetone phosphate (DHAP), its conversion to 1-O-acyl-DHAP, reduction of the oxo-group, and its acylation by acyl-CoA. Subsequent modifications give rise to plasmalogens in animals.

Although the presence of plasmalogens in anaerobic bacteria has been known for nearly 50 years (Allison et al. 1962; Wegner and Foster 1963; Goldfine 1964) and was described many times (Baumann et al. 1965; Hagen and Goldfine 1967; Koga and Goldfine 1984; MacDonald and Goldfine 1990; Silber et al. 1980; Prins et al. 1974; Watanabe et al. 1984; Paltauf 1983), their biosynthesis, which differs from that taking place in animals, is virtually unknown. It does not involve DHAP (see Fig. 2); this has been documented in *Clostridium beijerinckii* (Hill and Lands 1970), in which the $^{3}H/^{14}C$ ratios of diacyl phospholipids (PLs) and plasmalogens after addition of [2-^{3}H] glycerol and [1-^{14}C] glycerol into the growth medium were nearly identical. Should DHAP serve as an intermediate, then tritium could not be present on the C-2 of glycerol. Similar data were obtained with the Gram-negative anaerobes *Megasphaera elsdenii* and *Veillonella parvula* and anaerobic protists *Isotricha prostoma* and *Dasytricha ruminantium* (Prins and Van Golde 1976). These data imply that the difference between the organisms that use DHAP for plasmalogen biosynthesis and those that do not is not based on their eukaryotic or prokaryotic nature, but rather on whether they are aerobic or anaerobic (Prins and Van Golde 1976). Precursors for plasmalogen synthesis are assumed to be diacyl phospholipids (Hill and Lands 1970). A recent study (Guan et al. 2011)

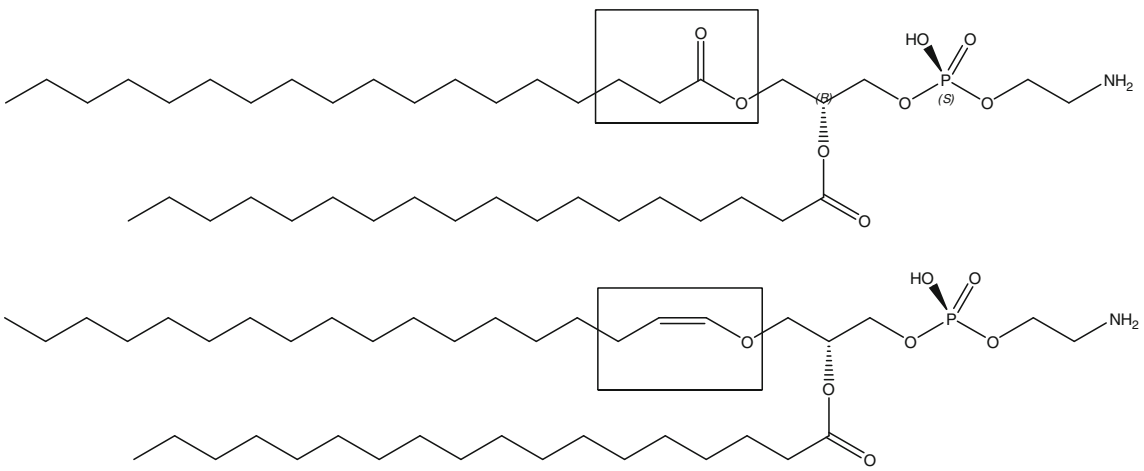

Fig. 1 Difference in the structures of diacyl and alkenylacyl ethanolamine

confirmed that plasmalogens are formed from diacylated phospholipids at a late stage of phospholipid formation in *Clostridium* species. Long-chain alcohols are incorporated into the plasmalogens of bacteria only to a limited extent (Paltauf 1983; Goldfine and Hagen 1972), whereas long-chain aldehydes and fatty acids were found to be incorporated into, e.g., *C. beijerinckii* plasmalogens (Baumann et al. 1965; Hagen and Goldfine 1967). The picture of plasmalogen biosynthesis in anaerobic bacteria, emerging from all these studies, is still incomplete and partly speculative (Fig. 2). The anaerobic biosynthesis of plasmalogens in animals, but not in aerobic

Table 1 Plasmenyl and plasmanyl lipids in bacteria

Bacterial strains	Plasmenyl[a]	Plasmanyl[b]
Anaerovibrio lipolytica L 1741	+[c]	ND[d]
Bacillus firmus duramycin-resistant mutant	+[e]	ND
B. firmus OF4 wild type	+ (trace)[e]	ND
Bacteroides ruminicola	+	ND
Bacteroides succinogenes	+[f]	ND
Clostridium sp. (3-97)	+	ND
Clostridium sp. (4-52)	+	ND
Clostridium acetobutylicum 179-121	+	+
C. acetobutylicum 314-48	+	ND
C. acetobutylicum DSM 1731	+[g]	ND
Clostridium butyricum	+[h]	ND
Clostridium innocuum ATCC 14501	+[i]	ND
Clostridium kainantoi	+	ND
Clostridium kaneboi	+	+
Clostridium novyi ATCC 27606	+[j]	ND
Clostridium perfringens	+	+
Clostridium saccharoperbutylacetonicum	+	+
Clostridium tetani ATCC 454	+[k]	ND
Desulforhabdus amnigenus	ND	+[l]
Desulfosarcina variabilis	ND	+[l]
Desulfovibrio sp.	+	ND
Megasphaera cerevisiae	+[j]	ND
Megasphaera elsdenii	+	ND
M. elsdenii B 159	+[c]	ND
M. elsdenii	+[m]	ND
M. elsdenii	+[n]	ND
Mycoplasma sp. (14 strains)	+[o]	ND
Mycoplasma fermentans	+[p]	+[p]
Myxococcus xanthus (8 strains)	+[q]	ND
Pectinatus cerevisiiphilus	+[m]	ND
Pectinatus frisingensis	+[m]	ND
Propionibacterium freudenreichii anaerobic culture	+	+
P. freudenreichii standing culture	+	+
Propionibacterium propionicum PCM 2431 (ATCC 14157T)	−	+[r]
Propionibacterium shermanii anaerobic culture	+	+
P. shermanii standing culture	+	+
Ruminococcus flavefaciens	+[s]	ND
Selenomonas ruminantium	+[t]	+
S. ruminantium glucose grown	+	+
S. ruminantium lactate grown	+	+
Treponema hyodysenteriae B78	+[u]	ND
Treponema pallidum (Reiter)	+[v]	ND
Veillonella gazogenes	+	+
Veillonella parvula ATCC 10790	+[c]	ND

[a] Kamio et al. (1969)

[b] Determined by TLC (Kim et al. 1970) with chimyl alcohol as internal standard

[c] Verkley et al. (1975) (plasmalogens of PlsPE were identified as dimethylacetals by GLC)

[d] Not determined

[e] Clejan el al. (1989)

[f] Wegner and Foster (1963)

[g] Oulevey et al. (1986) (the molar ratios of alkenyl group/lipid phosphorus was from 0.57 to 1.26 in polar lipids, i.e., PG, CL, monoglycosyldiglyceride, and diglycosyldiglyceride of this strain)

[h] Baumann et al. (1965)

[i] Johnston et al. (1994) (complex lipids, as e.g., D-Glcp(αl-3)radyl$_2$Gro and D-Galp(αl-2)D-Glcp(αl-3)radyl$_2$Gro, etc. contained l-*O*-(alk-l-enyl)-2-*O*-acyl species including diacyl species, determined by GC-MS and NMR)

[j] Guan et al. (2011) (the three phospholipids, i.e., PE (25 %), PG, and CL (43 %) were identified as plasmalogens, and their content (percent) was found by radiolabeling of [^{14}C]-labeled lipids)

[k] Johnston et al. (2010) (PE, PG, CL, and EtnPGlcNAcdiradylglycerol were identified by LC-MS, high-resolution ESI/MS, and NMR)

[l] Rutters et al. (2001)

[m] Helander and Haikara (1995)

[n] Van Golde et al. (1973) (content of PlsPS or PlsPE ranged from 70 to 90 % of total PS or PE, respectively, and measured by phosphorus analysis or ^{32}P analysis)

[o] Worliczek et al. (2007) (dimethylacetals were identified in 14 of 16 genera of *Mycoplasma*, which indicates the presence of plasmalogens)

[p] Wagner et al. (2000) (the presence of alkyl and vinyl (alk-1′-enyl) ether lipids in the cell membrane of aerobically grown *Mycoplasma fermentans* was proven by GC, MS, and NMR)

[q] Ring et al. (2006) (1-*O*-(13-methyl-1-*Z*-tetradecenyl)-2-*O*-(13-methyltetradecanoyl)-glycero-3-phosphatidylethanolamine and 1,2-di-(13-methyltetradecanoyl)-3-(13-methyltetradecyl) glycerols were identified by LC-MS and GC-MS, respectively)

[r] Pasciak et al. (2003) (mono- and di-glucosyl ether (plasmanyl) lipids identified by MS and NMR)

[s] Allison et al. (1962)

[t] Takatsuka and Kamio (2004)

[u] Matthews et al. (1980)

[v] Meyer and Meyer (1971)

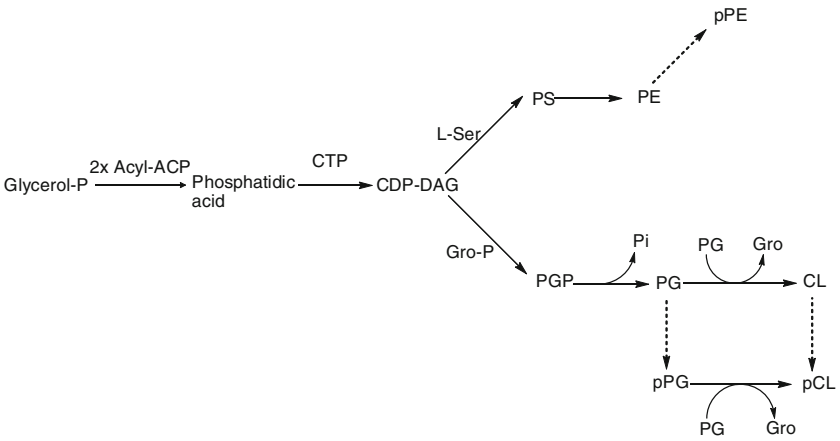

Fig. 2 A proposed anaerobic pathway for plasmalogen biosynthesis in bacteria. The number of steps leading from the diacyl phospholipids to the plasmalogens is unknown (Goldfine 2010)

bacteria, fungi, and plants was replaced by an aerobic pathway later in evolution.

This review does not deal with saturated ether lipids. These lipids, sometime called plasmanyl lipids, are present in bacteria as well as in archaebacteria (see the general structure in Fig. 3) (Koga and Morii 2007; Koga et al. 1993). This group of lipids contains some highly specific and unique polar lipids having 2,3-dialkyl-*sn*-glycerol backbones, whose stereochemistry is the opposite of that found in the two other primary kingdoms: bacteria (eubacteria) and eucarya (eukaryotes). Side chains are based on an isoprenoid chain, e.g., 2,3-diphytanyl-*O*-*sn*-glycerol (archaeol). Many lipids of this group contain also macrocyclic diethers. Another very curious group, which we mention only to illustrate some interesting features of metabolism of some extremophilic bacteria, is ladderane lipids (Damsté et al. 2005; Hopmans et al. 2006; Lanekoff and Karlsson 2010). Anammox bacteria contain ladderane fatty acids, i.e., acids with cyclobutane rings (Fig. 4).

Though the anaerobic mechanism of plasmalogen biosynthesis still present in contemporary anaerobic bacteria and in some anaerobic protozoa (Prins and Van Golde 1976) has switched to an aerobic mechanism found in oxygen-tolerant eukaryotes, aerobic bacteria retain almost all anaerobic prokaryotic biosynthetic mechanisms (Goldfine and Bloch 1963; Bloch 1994), e.g., the biosynthesis of monounsaturated fatty acids (Goldfine and Bloch 1961; Rock and Jackowski 2002). In plants, fungi and animals unsaturated fatty acids are formed by an oxidative mechanism, in which two hydrogen atoms are abstracted from long-chain saturated fatty acids (Bloomfield and Bloch 1960).

While most eukaryotes make plasmalogens by an oxidative mechanism, the biosynthesis of plasmalogens in anaerobes proceeds again without molecular oxygen. Though plasmalogens are readily degraded by reactive oxygen species (ROS), leading to the generation of *sn*-1-lyso-phospholipids whose accumulation can be toxic to cells (Morand et al. 1988; Marmer et al. 1986), they are abundant in ROS-producing animal cells, where they may serve as antioxidants (Morand et al. 1988). Bacteria rich in plasmalogens probably did not have the eukaryotic mechanisms for acylation of the resulting free hydroxyl group at the *sn*-1 position. All studied contemporary anaerobes have polar lipids with acyl chains at the *sn*-1 position analogous to their plasmalogens, for example, phosphatidylethanolamines (PE), PG, and cardiolipin. When plasmalogens are lost, membrane spaces are filled by these lipids.

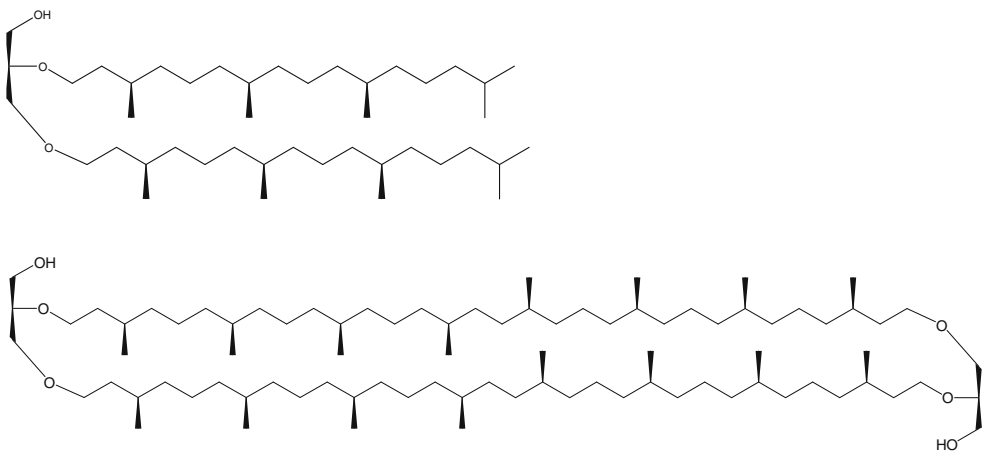

Fig. 3 Some major lipids of archaebacteria

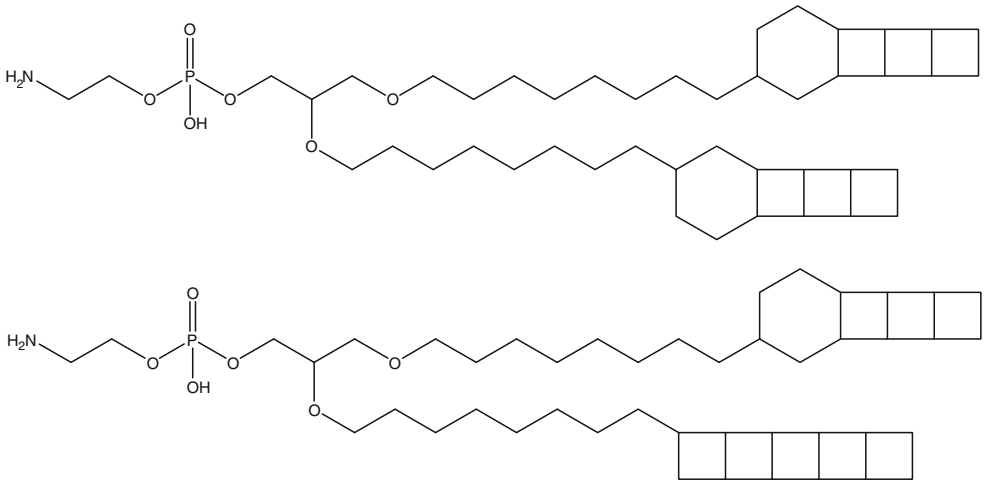

Fig. 4 Some major lipids of anammox bacteria

Analysis

Extraction of plasmalogen lipids proceeds always simultaneously with the extraction of other lipids (Kates 1986 and papers cited therein) and usually presents no problems. Much greater problems are encountered in the mutual separation of alkylacyl (plasmanyl), alkenylacyl (plasmenyl), and diacyl lipids.

Plasmalogens are generally taken to be susceptible to oxidative modifications at the *sn*-1 position (Catala 2009), and the extraction should, therefore, be performed in the presence of antioxidants, such as butylated hydroxytoluene (BHT). Interestingly, none of the many studies, describing the isolation of bacterial plasmalogens, has reported on the use of antioxidants to prevent the oxidation of the vinyl double bond (Bollinger et al. 2010; Johnston and Goldfine 1992; Kaufman et al. 1988; Johnston and Goldfine 1982; Khuller and Goldfine 1974; Lee et al. 1998; Guan et al. 2011). Yet, when plasmalogens were isolated from the tissues of warm-blooded vertebrates, for instance, rat erythrocytes and cerebellum, human erythrocytes and plasma, bovine heart, canine sarcolemma and chicken breast muscles, antioxidants (6-di-tert-butyl-p-cresol or BHT) were added, or the extraction was performed in a nitrogen atmosphere. However, it should be noted that these antioxidants were added to prevent the oxidation of polyunsaturated fatty acids rather than to prevent the oxidation of a double bond (Guan et al. 2001; Gross 1984; Hui et al. 2011; Mawatari et al. 2007).

The use of standards is generally fraught with problems since only two of them are commercially available, viz, 1-(1*Z*-octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphocholine and 1-(1*Z*-octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine. Moreover, these molecular species are commonly found in bacteria and also in other organisms (mammals, etc.). Standards with an odd number of carbon atoms in any of the two chains are not commercially available, and bacteria commonly contain odd-numbered molecular species. They can be synthesized—see, e.g., Hui et al. (2011), who synthesized 1-*O*-1′-(*Z*)-tricosenyl-2-oleoyl-*rac*-glycero-3-phosphocholine—but the synthesis is not trivial (seven reaction steps) and, to our mind, largely impracticable for most potential authors. Plasmalogens labeled with stable isotopes are also commercially unavailable, but can be synthesized (Reaxys 2012). All these facts obviously underlie the general lack of use of calibration standards and prevent the setting up of a calibration curve either in liquid chromatography–mass spectrometry (LC-MS) or tandem mass spectrometry (MS/MS).

Analysis of plasmalogen phospholipids makes use of their vinyl ether bond at the *sn*-1 position, which is labile to acid-catalyzed hydrolysis. The presence of mere traces of an acid causes hydrolysis, resulting in a lysophospholipid and a fatty aldehyde. Fast, simple, efficient, and sensitive (to 10^{-4}M sensitivity) determination of plasmalogens can be performed using the commercially available Purpald reagent (4-amino-5-hydrazino-4*H*-1,2,4-triazole-3-thiol), which forms purple-to-magenta-colored tetrazines with aldehydes.

Following their separation by, e.g., TLC, fatty aldehydes can be determined by GC-MS. Another possibility is the removal of the polar moiety of the molecule by phospholipase C and identification of alkylacyl, alkenylacyl, and diacylglycerols by HPLC (Guan et al. 2001; Nakagawa and Horrock 1983) or, after derivatization of the free hydroxyl group, by GC (Christie and Han 2010). All these methods are time and labor demanding. Another possibility, which has rather rarely been mentioned in the literature, is the separation of intact plasmalogens from diacylphospholipids by HPLC on diol columns (Uran et al. 2001). The authors (Olsson et al. 1996; Mawatari et al. 2007; Nguyen and Schug 2008) have always used a HPLC column with bonded stationary phase and a highly complex organic phase consisting of up to five solvents (Uran et al. 2001) and, in addition, elution with nonlinear gradient. The mobile

phase also contained a buffer composed of an organic acid (acetic or formic) and base (ammonia, ammonium acetate, or triethylamine). The elution order of individual phospholipids was different but, fortunately, plasmalogen was always eluted ahead of the corresponding diacyl derivative.

Amphiphilic molecules, such as phospholipids, can be separated on a hydrophilic interaction liquid chromatography (HILIC) column, although it has more often been used for separating water-soluble analytes (Nguyen and Schug 2008). Separation of PLs on HILIC column is described in only a few papers, with relatively controversial results concerning especially the elution of individual PL classes. Thus, Schwalbe-Herrmann et al. (2010) have described an elution order of PG, PE, and phosphatidylcholine (PC), whereas Kamleh et al. (2008) reported on the order PC, PS, and PG, and Scherer et al. (2010) obtained the sequence PG and PA.

Mass spectrometry is the most important method for structural characterization of all classes of glycerolipids. Modern soft ionization techniques, i.e., the formation of gas-phase ions without extensive fragmentation, such as fast atom bombardment (FAB), matrix-assisted laser desorption ionization, or electrospray ionization (ESI), allow an analysis of intact lipids. These lipids are complex mixtures which differ in fatty acyl or alkyl substituents to the glycerol backbone, and of course, there are also different substituents in the *sn*-3 position of glycerol, which is bound to polar groups, see Fig. 1.

Soft ionization techniques generate the majority of ions, such as $[M+H]^+$, $[M+NH_4]^+$, or $[M^+$ alkali metal ions (Li, Na, or K)$]^+$ for positive ion mode, and ions $[M\text{-}H]^-$ in case of negative ion mode. There are two basic methods of analysis. The first one uses the front-end HPLC apparatus, which separates glycerolipids into subclasses by normal phase, and these are further analyzed by MS, or can be divided by reversed phase into the molecular species (not always with complete success) and then analyzed using the MS (or tandem MS).

Shotgun lipidomic approaches have also been described for glycerolipid analysis without prior chromatographic separation, i.e., the direct injection of the mixture of lipids for MS and their analysis, in most cases, by tandem MS.

Hsu and Turk (2007) reported on ESI-MS or ESI-MS/MS identification of intact plasmalogens as lithiated adducts, e.g., $[M+Li]^+$ molecular ions and their cleavage to give characteristic fragments that allow identification of individual molecular species. In the presence of lithium salts, plasmalogens form $[M+Li]^+$ ions which, upon CID, produce product ion spectra that contain diagnostic fragment ions for both the polar head and the fatty acyl (alkyl) groups.

Table 2 provides illustrative cases for LC-MS methods and for direct MS analysis, which are used for the separation and identification of plasmalogens in microorganisms. Two methods were used basically for analysis, i.e., FAB and ESI, both of them in the positive and/or in the negative mode. The FAB method was used mainly in the last millennium, while the ESI methods have been used in about the past 10 years. Since they were often not sufficiently efficient to yield a simple mass spectrum, it was necessary to use more sophisticated methods of analysis. A method of choice appears to be tandem MS, in some cases up to MS^4, which allowed, e.g., the identification of dozens of molecular species of plasmalogens in protozoa.

The use of the most advanced technology - nano-electrospray ionization tandem mass spectrometry (nano-ESI-MS) in low- or in high-resolution mode - enabled Richmond et al. (2010) to identify more than 500 molecular species of phospholipids, including dozens of plasmanyl and plasmenyl phospholipids. For example, the authors have identified plasmenyl-18:1/24:6 PC or plasmenyl-16:2/16:1 PI in *Trypanosoma brucei*.

In comparison with LC-MS analysis of lipids, shotgun lipidomics offer the possibility to acquire the mass spectrum featuring molecular ions of individual molecular species of the lipid at a constant concentration of the solution during direct infusion. Precursor ion scans and/or neutral loss scans of the individual lipid molecular species can then be used for their identification and quantification without the time constraints common in the analysis during chromatographic elution. Because the majority of lipid species represent linear combinations of polar head groups or fatty acids, each series of scans then determines the identity of the molecular ion.

On the other hand, the main advantage of LC/MS over MS/MS is that LC/MS or LC/MS/MS can be used for quantitative analysis of different type of lipids, differing chiefly in FAs; e.g., unsaturated fatty acids are separated by HPLC from cyclopropyl fatty acids. This separation and identification of unsaturated or cyclopropyl fatty acids cannot be performed with MS/MS, see e.g., the statement "indicating that the 17:1 fatty acid may represent a 9,10-methylene hexadecanoic acid, a cyclopropane fatty acid rather than a unsaturated fatty acid" in the paper of Hsu and Turk (2010).

Lipids containing isobaric FAs, e.g., straight chain FAs, iso-FAs, and anteiso-FAs as triacylglycerols (Schreiberova et al. 2010), which cannot be distinguished by MS or by tandem MS, can also be separated. Furthermore, HPLC can be used to separate molecular species of lipids containing positional isomers of fatty acids, such as oleic-vaccenic (Leskinen et al. 2010) and α- and γ-linolenic acids (Laakso 1997). Separation and identification of molecular species of lipids, containing α- and γ-linolenic acids, has a crucial effect on the utilization of these two acids as nutrients.

This can be illustrated by the following example. LC/ESI-MS^2 was used to analyze phospholipids from three

Table 2 Sources of plasmalogens, HPLC techniques, and analytical conditions used for their separation and identification

Literature	Column type	Mobile phase; temperature/infusion solvent/matrix	Flow (mL/min)	Ion source analyzer	Source conditions	Scan type	Mass range (*m/z*)	Samples matrix	Special prechromatography adjustment
Das and Hajra (1995)	Direct MS analysis	Nitrobenzyl alcohol matrix		FAB-MS-MA		+Mode		Synthetic plasmanyl LPC	
Morita et al. (2010)	Direct MS analysis	10 mM $AcONH_4$ in MeOH/$CHCl_3$ (75:25)		ESI-QqQ	4.5 kV	+Mode; TIC; precursor ion scan (184*m/z*); neutral ion scan (141*m/z*)	600–1,-00-0	Diradyl PC monitoring of PE *N*-methyltransferase activity	
Hsu and Turk (2007)	Direct MS analysis	MeOH		ESI-linear IT	300 °C, 4.5 kV	MS^3; MS^4		Diradyl PLs from *Leishmania major* and African trypanosomes	
Richmond et al. (2010)	Direct MS analysis			ESI-QqQ	+700 V, −900 V	Nano-ESI-MS-MS; precursor scanning for *m/z* 153	400–2,-00-0	*Trypanosoma brucei* cell culture	
Rutters et al. (2001)	Lichrosphere 100 diol 2× 125 mm; 5 μm	(1) hexane/*i*-PrOH/HCOOH/ NH_4OH (79:20:1.2:0.04) and (2) *i*-PrOH/water/HCOOH/NH_4OH (88:10:1.2:0.04); gradient	0.35	ESI-IT	200 °C, −4.5 kV	−Mode; MS^2; dependent scan mode		Plasmanyl PLs from sulfate-reducing bacteria *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*	Silica gel column chromatography
Hartvigsen et al. (2001)	ODS Hypersil 2.1×200 mm; 5 μm	MeOH/water/NH_4OH	0.40	ESI-Q	270 °C, −4 kV	+Mode; CID	100–1,-10-0	Synthetic mixtures of plasmanyl–glycerols	
Bollinger et al. (2010)	Zorbax SB-C8 2.1×50 mm; 5 μm	(1) MeOH/ACN/1 mM $AcONH_4$ (60:20:20) and (2) 1 mM $AcONH_4$ in EtOH; gradient; 30 °C	0.20	ESI-qTOF	−4.2 kV, +5.5 kV	±Mode; CID		Pls of PE, PG, CL, and *N*-acetylglucosaminyl diradylglycerol of *Clostridium tetani*	
Rezanka et al. (2011)	ZIC-HILIC 2.1×250 mm; 3.5 μm	(1) ACN/10 mM $HCONH_4$, 0.5 mM LiOH, and 0.5 mM AcOLi in water (80:20) and (2) ACN/20 mM $HCONH_4$, 0.5 mM LiOH, and 0.5 mM AcOLi in water (50:50); gradient; 50 °C	0.18	ESI-QqQ	300 °C, 3.9 kV	+Mode; TIC; CID	200–1,-10-0	Pls of PG, PE, PC, and PS from *Pectinatus*	
Guan et al. (2011)	Ascentis Si HPLC column, 25 cm× 2.1 mm, 5 μm,	(1) chloroform/methanol/ammonium hydroxide (800:195:5), (2) chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5), and (3) chloroform/methanol/water/ ammonium hydroxide (450:450:95:5); gradient	0.3	ESI-Q	−4.500 kV	−Mode, MS^2		PLs biosynthetic pathway in anaerobic *Clostridium novyi*	2D-TLC

species of the anaerobic beer-spoilage bacterium of genus *Pectinatus* (Rezanka et al. 2011). Analysis of total lipids on a HILIC column separated diacyl and plasmalogen phospholipids. Plasmalogens were then analyzed by means of the ESI-MS^2, and more than 220 molecular species of four classes of plasmalogens, i.e., PlsPC, PlsPE, PlsPG, and PlsPS, were identified. The method showed excellent reproducibility, high sensitivity (from 1 μmol/mL), and dynamic range (four orders of magnitude). In all classes of plasmalogens, phospholipids were eluted first and were followed by nearly baseline-separated corresponding diacyl phospholipids. The elution can conveniently be performed by an aqueous phase with a buffer compatible with ESI.

Simultaneous detection of intact plasmalogens, together with other phospholipid classes, having odd- or even-chain fatty acids or alkyls or alkenyls, provides a valuable tool for the study of phospholipids. The fragmentation processes observed by tandem MS of plasmalogens generate unique determinants of the identities of the 1-*O*-alk-1-enyl chains at the *sn*-1 position and the fatty acid esterified at the *sn*-2 position of the glycerol backbone.

Tandem MS of plasmalogen molecules as lithiated adduct ions is an optimum method for structural characterization. The structure of plasmalogen molecules can be easily determined from the positive ESI showing abundant fragment ions with different polar head groups, fatty acid constituents, and regiospecificity. The $[M+Li]^+$ ions of plasmalogens that have undergone consecutive losses of the fatty acid substituent at *sn*-2 and the polar head group, i.e., the $[M+Li–R_2COOH–$ polar head group$]^+$ ions, are very useful in differentiating the plasmenyl and diacyl phospholipid subclasses. Thus, structures of plasmalogens with isobaric isomers in mixtures can be unambiguously determined (Hsu and Turk 2005, 2007).

Conclusion

The future of plasmalogen analysis can be seen in two areas. The first is the use of new ionization techniques, such as, e.g., atmospheric pressure photoionization mass spectrometry, MS/MS combination, especially when high-resolution mass spectrometry by numerous time-of-flight mass spectrometers is used, and also the use of ultra-performance liquid chromatography. The second area offers the possibility of analyzing a much larger spectrum of samples. To our knowledge, LC-MS and direct inlet to mass spectrometer has not yet been used for analyzing materials from various bacteria and/or protozoa containing unusual FAs, etc. We foresee explosive development in this area in the next decade.

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