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Review

Plasmalogens - Ubiquitous molecules occurring widely, from anaerobic bacteria to humans

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A B S T R A C T

Plasmalogens are a group of lipids mainly found in the cell membranes. They occur in anaerobic bacteria and in some protozoa, invertebrates and vertebrates, including humans. Their occurrence in plants and fungi is controversial. They can protect cells from damage by reactive oxygen species, protect other phospholipids or lipoprotein particles against oxidative stress, and have been implicated as signaling molecules and modulators of membrane dynamics. Biosynthesis in anaerobic and aerobic organisms occurs by different pathways, and the main biosynthetic pathway in anaerobic bacteria was clarified only this year (2021). Many different analytical techniques have been used for plasmalogen analysis, some of which are detailed below. These can be divided into two groups: shotgun lipidomics, or electrospray ionization mass spectrometry in combination with high performance liquid chromatography (LC-MS). The advantages and limitations of both techniques are discussed here, using examples from anaerobic bacteria to specialized mammalian (human) organs.

1. Introduction

Plasmalogens, 1-O-alk-1′-enyl, 2-acyl glycerolipids, are one of the most types of phospholipids occurring widely in nature. They are found in anaerobic bacteria, myxobacteria, invertebrates, vertebrates, and protozoa [1]. Their presence or absence is shown in Table 1. With a few exceptions, they are not found in fungi [1] and their presence in plants has been reported [2,3], but not confirmed [4].

Plasmalogens are glycerol derivatives that are characterized by the presence of a vinyl ether bond at the *sn*-1 position and an ester bond at the *sn*-2 position (Fig. 1) [5]. At the *sn*-1 position is a long-chain alcohol, which has predominantly 16 and/or 18 carbon atoms, but in bacteria there can also be odd and branched chains [6,7].

The presence of ether lipids in anaerobic bacteria was first observed in the 1960s, in the Gram-negative rumen bacteria, *Ruminococcus avefaciens* [8] and *Bacteroides succinogenes* [9] and then in Gram-positive *Clostridium beijerinckii* (formerly *Clostridium butyricum,* [10]) [11]. Since then, plasmalogens have been found in a wide variety of anaerobic bacteria [12].

The most common phospholipids in bacteria are phosphatidylethanolamine (PE), phosphatidylglycerol (PG), phosphatidylserine (PS) and cardiolipin (CL) and their plasmalogen forms (P-PE, P-PG, P-PS, and P-CL). *N*-methyl-PE and its plasmalogen derivatives have been found in *Clostridium beijerinckii* and *C. saccharoperbutylacetonicum* [13] and aminoacyl-PG is present in *C. perfringens* [14,15] and *C. novyi*. Glycosyldiaradylglycerols have been found in a number of species [13]. Plasmalogen-PE and the plasmalogen form of phosphatidylcholine are most commonly found in animal tissues. Decreased plasmalogen levels, or even a complete absence, are associated with several neurological disorders including Alzheimer's disease, Zellweger's syndrome, and

Abbreviations: ACBD, acyl-CoA-binding domain; APCI, atmospheric pressure chemical ionization; A-PE, 1-alkyl-2-acyl phosphatidylethanolamine; BLAST, Basic Local Alignment Search Tool; CL, cardiolipin; DHAP, dihydroxyacetone phosphate; DRG, diradylglycerol; ELSD, evaporative light scattering detection; ESI, electrospray ionization; FA, fatty acid; PUFA, polyunsaturated fatty acids; FAME, fatty acid methyl esters; GC, gas chromatography; HILIC, hydrophilic interaction liquid chromatography; HPLC, High Performance Liquid Chromatography; IPTG, isopropyl β-d-1-thiogalactopyranoside; LC, liquid chromatography; MALDI, matrix-assisted laser desorption ionization; MHDRG, monohexosyldiradylglycerol; MHMAG, monohexosyl monoacylglycerol; MS, mass spectrometry; NGF, nerve growth factor; NPLC, normal phase liquid chromatography; PA, phosphatidic acid; P-CL, 1-alkenyl-2-acyl cardiolipin; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; P-PC, 1-alkenyl-2-acyl phosphatidylcholine; P-PE, 1-alkenyl-2-acyl phosphatidylethanolamine; P-PS, 1-alkenyl-2-acyl phosphatidylserine; PS, phosphatidylserine; PT, phosphatidylthreonine; TAG, triacylglyceride; Q-TOF, quadrupole time-of-flight; RP, reverse phase; TLC, thin layer chromatography; UHPLC, ultra-high-pressure liquid chromatography; VAP, vesicle-associated membrane protein;.

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Table 1
Plasmenyl and plasmanyl lipids in bacteria and invertebrates.

Organism	Plasmenyl [109]	Plasmanyl [110]
Anaerovibrio lipolytica L 1741	+ [111]	ND
Bacillus firmus duramycin-resistant mutant	+ [112]	ND
B. firmus OF4 wild type	+(trace) [112]	ND
B. succinogenes	+ [9]	ND
bacteria?	- [113]	+
Bacteroides ruminicola	+	ND
Berenicea meandrina	+ [9]	–
Bifidobacterium animalis	- [91]	–
B. animalis subsp. *lactis*	+ [114]	–
B. longum	+ [91]	–
Clostridium sp. (3–97)	+ [110]	ND
Clostridium sp. (4–52)	+ [110]	ND
C. acetobutylicum 179–121	+	+
C. acetobutylicum 314–48	+	ND
C. acetobutylicum DSM 1731	+ [115]	ND
C. butyricum	+ [24]	ND
C. cadaveris	+ [89]	–
C. fallax	+ [89]	–
C. innocuum ATCC 14501	+ [116]	ND
C. kainantoi	+	ND
C. kaneboi	+	+
C. novyi ATCC 27606	+ [90]	ND
C. novyi	+ [90]	–
C. perfringens	+	+
C. perfringens	+ [31]	–
C. saccharoperbutylacetonicum	+	+
C. tetani ATCC 454	+ [92]	ND
C. tetani	+ [92]	–
Dendrobeania flustroides	+ [82]	–
Desulforhabdus amnigenus	ND	+ [117]
Desulfosarcina variabilis	ND	+ [117]
Desulfovibrio sp.	+	ND
Eudistoma sp.	+ [87]	–
Chlamydia	+ [93]	–
Leishmania major	+ [118]	–
Leptoclinides uniorbis	+ [87]	–
Megasphaera cerevisiae	+ [90]	ND
M. elsdenii	+	ND
M. elsdenii	+ [119]	ND
M. elsdenii	+ [120]	ND
M. elsdenii B 159	+ [111]	ND
Mycoplasma fermentans	+ [121]	+ [121]
Mycoplasma sp. (14 strains)	+ [122]	ND
Myxococcus xanthus (8 strains)	+ [106]	ND
M. xanthus	+ [21]	–
M. xanthus	+ [123]	–
Pectinatus cerevisiiphilus	+ [119]	ND
P. freudenreichii standing culture	+	+
P. frisingensis	+ [119]	ND
P. propionicum PCM 2431 (ATCC 14157 T)	–	+ [124]
P. shermanii anaerobic culture	+	+
P. shermanii standing culture	+	+
Propionibacterium freudenreichii anaerobic culture	+	+
Romboutsia sp.	- [125]	–
R. ilealis	- [125]	–
R. lituseburensis	- [125]	–
Ruminococcus flavefaciens	+ [126]	ND
S. ruminantium glucose-grown	+	+
S. ruminantium lactate-grown	+	+
Selenomonas ruminantium	+ [127]	+
Stigmatella aurantiaca	+ [123]	–
T. pallidum (Reiter)	+ [128]	ND
Treponema hyodysenteria B78	+ [129]	ND
V. parvula ATCC 10790	+ [111]	ND
Veillonella gazogenes	+	+

ND = not detected.

Niemann-Pick type C disease [16–18]. Molecular functions of ether lipids are the focus of a review of Jimenez-Rojo and Riezman [19]. The regulation of plasmalogen biosynthesis and its physiological role in mammals, i.e., plasmalogen homeostasis, is described in the review of Honsho and Fujiki [20].

2. Biosynthesis in aerobic organisms

The biosynthesis of glycerol ethers, including plasmalogens, has been studied mainly in mammalian tissues and, in contrast to diacylphospholipids, proceeds quite differently. The biosynthetic pathway was first delineated enzymatically in the peroxisomal membrane followed by biosynthesis in the endoplasmic reticulum (Fig. 2). The long chain fatty alcohol is first synthesized from the appropriate long chain FA by means of fatty acyl-CoA reductase. In the peroxisome, dihydroxyacetone phosphate (DHAP) is first esterified with a long chain acyl-CoA, which is then replaced by a long chain fatty alcohol. This reaction is catalyzed before the ether linkage is introduced by replacing the acyl group with a long chain alcohol; this reaction is catalyzed by alkyl-DHAP synthase (alkyl-dihydroxyacetonephosphate synthase). The next step is to transfer the respective intermediate from the peroxisome to the endoplasmic reticulum, catalyzed by the protein ACBD5, which, depending on the isoform, e.g. in pigs, is about 500 amino acids in length. It binds to another protein (VAP-B) on the cytosolic side of the endoplasmic reticulum, and the VAP-ACBD5 complex facilitates the association of the two organelles. A further reaction step, already in the endoplasmic reticulum, is the reduction of the oxo (keto) group in the *sn*-1 position to 1-alkyl-*sn*-glycero-3-phosphate and its further reaction gives 1-alkyl-2-acyl-*sn*-glycero-3-phosphate. This reaction is catalyzed by alkyl/acyl-glycero-3-phosphate acyltransferase. Hydrolysis of the polar head group (phosphate group) by phosphohydrolase leads to 1-alkyl-2-acyl-*sn*-glycerol. Similarly to diacyl phospholipids, the conversion of 1-alkyl-2-acyl-*sn*-glycerol is catalyzed by the same enzymatic system. A key step in plasmalogen biosynthesis is the oxygen-dependent Δ1′-desaturase reaction, which results in the formation of plasmalogens such as 1-alk-1′-enyl-2-acyl-*sn*-PE. This enzyme has been identified in two completely different organisms, i.e. in humans as TMEM189 and in the aerobic bacterium *Myxococcus xanthus* as CarF [21].

Other phospholipids are synthesized in the usual ways; plasmenylglycerol is formed by hydrolysis of P-PE and by choline phosphotransferase and is then converted to P-PC. Similarly, P-PC can be synthesized via an alternative pathway, where P-PE is gradually methylated by *N*-methyltransferase to P-PC. Further modifications of the polar head or fatty acyls in the *sn*-2 position no longer affect the substituent in the *sn*-1 position and it remains unchanged. As an aside, the polar head at the *sn*-3 position can be exchanged by fatty acids. This reaction gives 1-alk-1'-enyl-2,3-diacyl-*sn*-glycerols.

3. Biosynthesis in anaerobic organisms

Almost 50 years ago, the biosynthesis of plasmalogens in animal tissues was found to require molecular oxygen [22]. For this reason, biosynthesis in anaerobic bacteria must be completely different. Goldfine [23] showed that proliferating bacteria of the genus *Clostridium* convert the *sn*-1-acyl chain to alk-1-enyl ether. Inhibition of phosphatidylserine decarboxylase by hydroxylamine in proliferating *C. butyricum* (now *C. beijerinckii*) resulted in 95% diacyl-PS and only 5% P-PS. After hydroxylamine removal, PE biosynthesis proceeded rapidly and P-PE was produced thereafter.

An experiment with *C. beijerinckii,* adding radioactive inorganic phosphate (^{32}P) to the culture medium, showed that the incorporation of ^{32}P diacyl-PE and diacyl *N*-methyl-PE delayed the formation of the respective plasmalogens by about 7 min [24]. Furthermore, the addition of ^{14}C acetate to a culture of the same bacterium was consistent with the precursor relationship between diacyl-phospholipids and plasmalogen phospholipids [25]. The same results were observed for *C. butyricum,* where the PE designation preceded the P-PE, P-PG and/or P-CL [26]. Analysis of aldehydes and *sn*-1 and *sn*-2 acyl chains in *C. beijerinckii* after acid hydrolysis of phosphatidylethanolamines also revealed a great similarity in aldehyde structures as with fatty acids (Table 2) [25].

The bacterium *C. beijerinckii* was cultured under anaerobic conditions at 37 °C in tryptone-glucose-yeast extract medium (consisting of 30 g/L tryptone, 20 g/L glucose, 10 g/L yeast extract and 1 g/L L-cysteine). The cultures grew to an A_{600} of 0.30–0.35 and commercially acquired 17:0/17:0-PS was added at this time. Samples were collected at one hourly intervals and the appropriate molecular species (di-17:0-PE, di-17:0-*N*-methyl-PE and plasmalogens di-17:0-PE) were identified by MS [25]. Because *C. beijerinckii* does not contain phospholipids of molecular structure 17:0/17:0, the only source must be an exogenous substrate [27].

Experiments with labeled [2−^{3}H] and [1−^{14}C] glycerol in the culture medium of *C. beijerinckii* showed that the ^{3}H/^{14}C ratios of the resulting diacylphospholipids and plasmalogen-phospholipids were almost identical. This experiment showed that dihydroxyacetone phosphate is not an intermediate in biosynthesis, as tritium on C-2 glycerol could not be present. Further experiments were performed with Gram-negative anaerobes *Megasphaera elsdenii* and *Veillonella parvulla* and with anaerobic protozoa *Isotricha prostoma* and *Dasytricha ruminantium*.The results confirmed that the dividing line between organisms that use dihydroxyacetone phosphate for plasmalogen biosynthesis and those that do not are not eukaryotic-prokaryotes, but rather aerobic-anaerobic organisms [28].

Further experiments were completed to determine whether biosynthesis of plasmalogens could be performed with long chain alcohols. Their incorporation into anaerobic bacteria was very low, in contrast in eukaryotic organisms [29,30]. Specifically, [1-^{3}H, 1−^{14}C] palmitaldehyde was incorporated into *C. beijerinckii* and approximately 15% of tritium was found to be contained in alk-1′-enyl chains, indicating that the aldehyde was not oxidized to FA prior to incorporation [25].

A recently published research article has finally elucidated the biosynthesis of plasmalogens in anaerobic bacteria. To verify plasmalogen production under anaerobic conditions, the authors [31] used *Escherichia coli,* which is itself free of plasmalogen production, as the host strain. Into *E. coli* was cloned the *pls* operon and the strain was cultured under anaerobic conditions. Plasmalogen production was recorded before and after induction by IPTG (isopropyl β-d-1-thiogalactopyranoside, i.e., the inducer of the *pls* operon in the recombinant strain). The authors detected plasmalogen production in the recombinant strain without an IPTG inducer under anaerobic conditions. They performed the same experiment with the recombinant strain under aerobic conditions and did not detect any plasmalogen production. This demonstrated the effect of oxygen on plasmalogen production. Furthermore, their Fig. 3 shows the presence of selected genes from the *pls* operon (Pfam domain) occurring in selected bacteria of the intestinal microbiome. A complete list of many hundreds of bacteria is given as a supplement (Excel spreadsheet) to their paper. The authors of this work selected two plasmalogen synthesizing genes (e.g., benzoyl-CoA reductase and 2-hydroxyglutaryl-CoA dehydratase), which correspond to PlsA or PlsR, respectively, and the presence of their genes was searched for in the microbiome. They found that plasmalogen positive and plasmalogen negative strains were present in the microbiome. Those that were plasmalogen positive, according to BLAST, carried gene sequences homologous to those in the *pls* operon and were grouped into Pfam domains. The plasmalogen Pfam domain exists in three sets of genes. Group 1 consists of a single gene encoding all four Pfam domains while groups 2 and 3 are composed of two genes, G1 and G2, with a variable number of domains.

4. Analysis of plasmalogens

Plasmalogens are usually extracted together with other lipids. Their extraction is usually straightforward, but their separation and identification can be problematic. Catala et al. [32] showed that alkenyl acyl lipids were sensitive to atmospheric oxygen; modifications at the *sn*-1 position occur. Furthermore, they are very easily hydrolyzed at acidic pH. The addition of antioxidants to the extraction of plasmalogens is not a common practice [33], although, in the isolation of plasmalogens from mammalian tissues, antioxidants have been used. However, we assume that these antioxidants were added to prevent oxidation of polyunsaturated fatty acids rather than to prevent double bond oxidation [33–36].

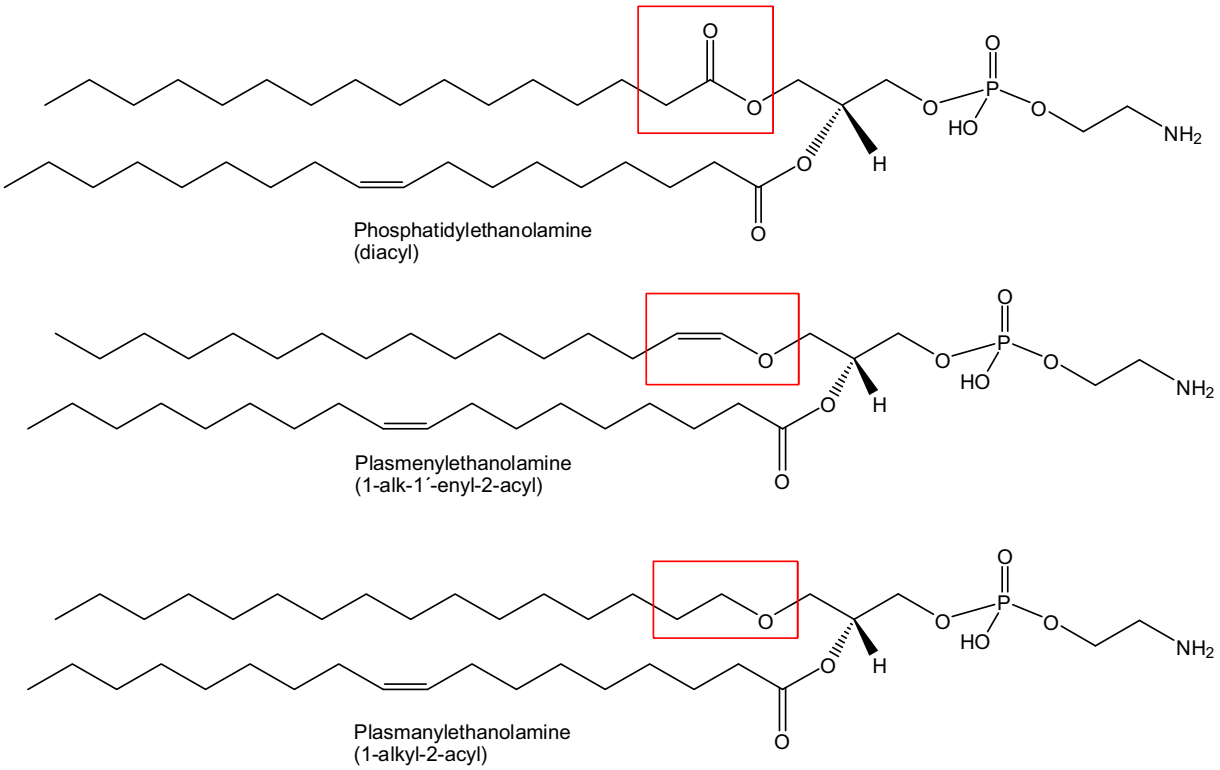


Fig. 1. Differences in the structures of alkenylacyl (plasmenyl), alkylacyl (plasmanyl), and diacyl ethanolamine.

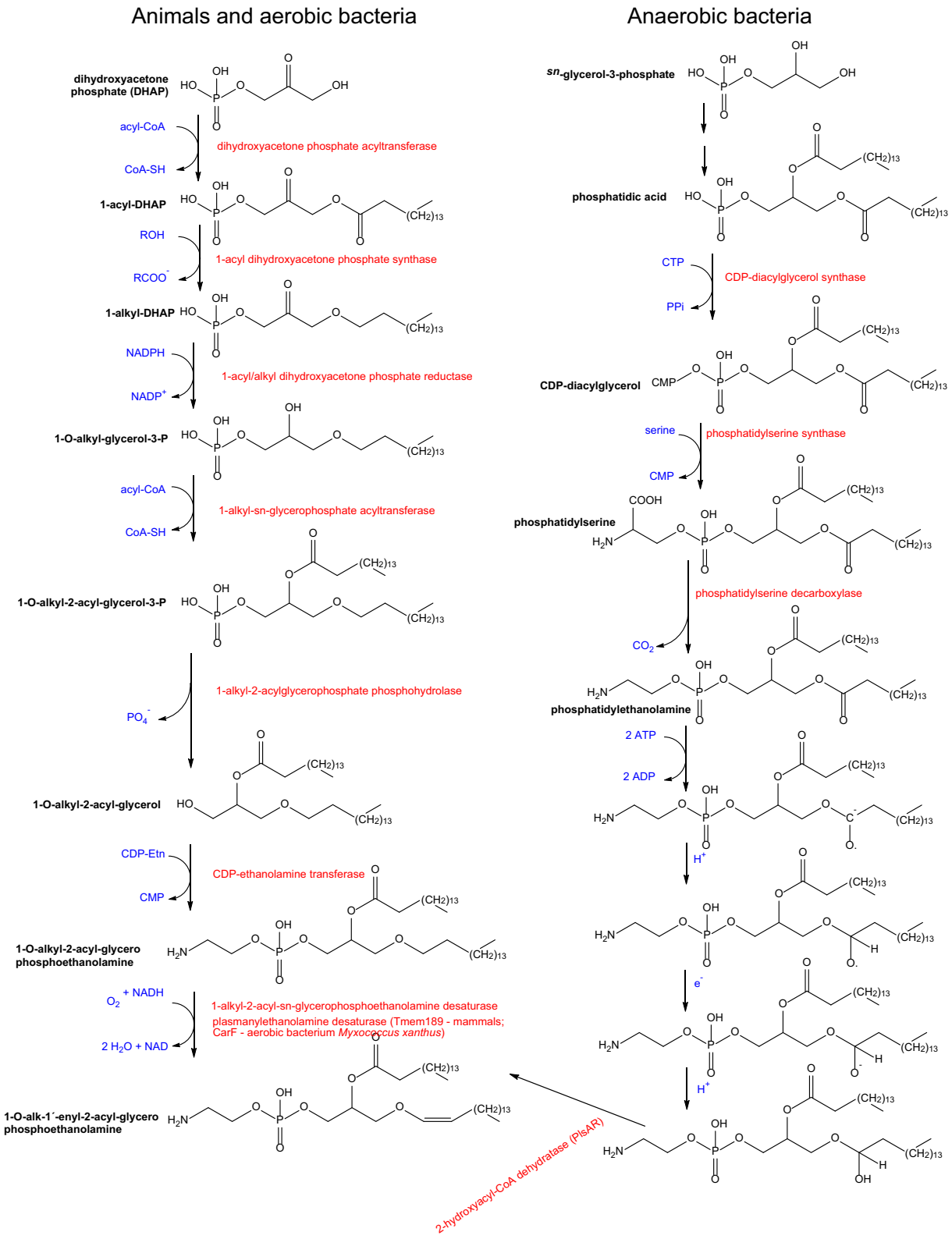


Fig. 2. Plasmalogen biosynthesis in aerobic and anaerobic organisms.

Another obstacle to the determination of plasmalogens is the general lack of commercially available standards. Unlike diacyl phospholipids, alkenyl acyl phospholipids are not readily available commercially. In contrast, all major classes of phospholipids are available; plasmalogens such as alkenyl acyl cardiolipins, to the best of our knowledge, are not available at all. Plasmalogens occurring in anaerobic bacteria, where chains of odd-numbered acyls or branched acyls (iso and/or anteiso) are often present, cannot be purchased. Standards labeled with stable isotopes, e.g., deuterium or ^{13}C are available. Examples are 1-(1*Z*-octadecenyl)-2-oleoyl(D9)-*sn*-glycero-3-phosphocholine or 1-(1*Z*-octadecenyl)-2-oleoyl(d9)-*sn*-glycero-3-phosphoethanolamine. As an example that has not yet been published, we show the tandem mass spectra of commercially

Table 2
Comparison of fatty aldehydes derived from the *sn*-1 alk-1′-enyl chain with fatty acyl chains of *C. beijerinckii* PE and P-PE [25].

Chain length: number of double bonds	Aldehydes *sn*-1	Fatty acids *sn*-1	Fatty acids *sn*-2
	Weight %		
14:0	<1	2	6
16:0	35	32	66
18:0	3	<1	<1
16:1 + 17:cyc	45	48	18
18:1 + 19:cyc	18	18	10
Total saturated	38	34	72
Total unsaturated[a]	63	66	28

[a] Includes cyclopropane aldehydes or fatty acids, which are derived from unsaturated chains.

available plasmalogen (1-(1Z-octadecenyl)-2-oleoyl(D9)-*sn*-glycero-3-phosphoethanolamine) and the tandem MS of plasmalogens (1-(1Z-octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine) from a previously published article [7], see Fig. 3. Of course, it is possible to synthesize any standard on your own, or to order, unfortunately these solution are often impossible, either due to lack of time or lack of funds.

4.1. Total organic synthesis

The possibilities of total organic synthesis of plasmalogens are mentioned briefly and generally. The starting material used is a glycerol derivative, i.e. 1,2-isopropylidene-*sn*-glycerol (2,2-dimethyl-1,3-dioxolane-4-methanol) [37,38] which is a commercially available compound. For the synthesis of isotopically labeled plasmalogen [39], $^{13}C_3$ glycerol was used. The basic scheme of synthesis is shown in Figs. 4 and 5. The reactions are always multistep and the overall yield after the fourteen step reaction was 39% (Table 4). A list of chemical compound abbreviations is given under the figures.

Several stereo selective reactions must be used in the organic synthesis of plasmalogens. One of them is the formation of the cis (*Z*) enol ether double bond. Another reaction is the stereo-selective protection of the OH group at the *sn*-2 position, or if plasmalogens containing a double bond in the acyl chain are synthesized, it is a selective reduction of the triple bond to the cis double bond, see [39]. In the case of the nine-step synthesis described by Van den Bossche et al. [40] two hydroxyls were protected in the starting compound and enol ether was formed in the next reaction. The next reaction step was the removal of both protecting groups and stereo selectively in the *sn*-2 position, i.e. the secondary hydroxyl was protected by another group (t-Bu_2-n-Bu-Si-). At the *sn*-3 position, the free hydroxyl reacted to form phosphocholine, which was converted to P-PC after reaction with the acid anhydride. The yield of all reaction steps was 12.4%. Where a P-PC containing an unsaturated acyl should be synthesized, for example, the procedure described in the [39] can be used or one of the commercially available PUFAs (arachidonic, eicosapentaenoic, docosahexaenoic, etc.) can be utilized. Both reaction schemes are listed as possibilities for total synthesis of plasmalogens, and partial synthesis has also been described previously [41].

4.2. Introduction to plasmalogen analysis

The vinyl ether bond at the *sn*-1 position is labile in an acidic environment and is readily hydrolyzed to form fatty aldehydes and lysophospholipids. A very sensitive test (~ 0.1 mM) for the presence of plasmenyl phospholipids in the sample uses Purpald reagent (4-amino-5-hydrazino-4*H*-1,2,4-triazole-3-thiol) which forms purple tetrazines in a reaction with aldehydes [42]. Fatty aldehydes can also be separated by TLC and identified by different chromatographic methods.

Free aldehydes and their dimethyl acetals can be determined colorimetrically as *p*-nitrophenylhydrazones at 395 nm. Phospholipase A (E. C.3.1.1.4) specifically hydrolyzes the fatty acid ester linkage in the *sn*-2 position of glycerophospholipid, including plasmalogen, to form a free fatty acid and the lysophosphatide. The basic analysis of plasmalogens involves the hydrolysis of both acyl and alkenyl chains. While acyl chains

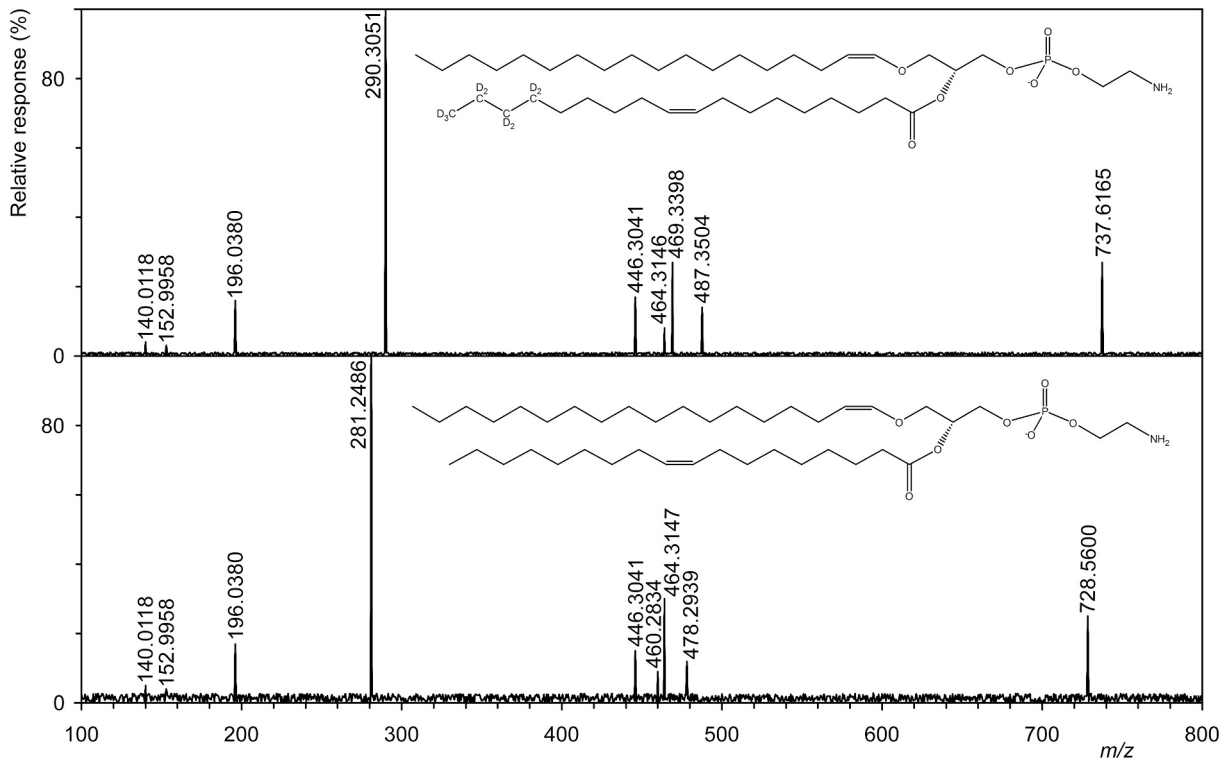


Fig. 3. The tandem mass spectra of commercially available plasmalogen (1-(1Z-octadecenyl)-2-oleoyl-(D9)-*sn*-glycero-3-phosphoethanolamine) and (1-(1Z-octadecenyl)-2-oleoyl-*sn*-glycero-3-phosphoethanolamine).

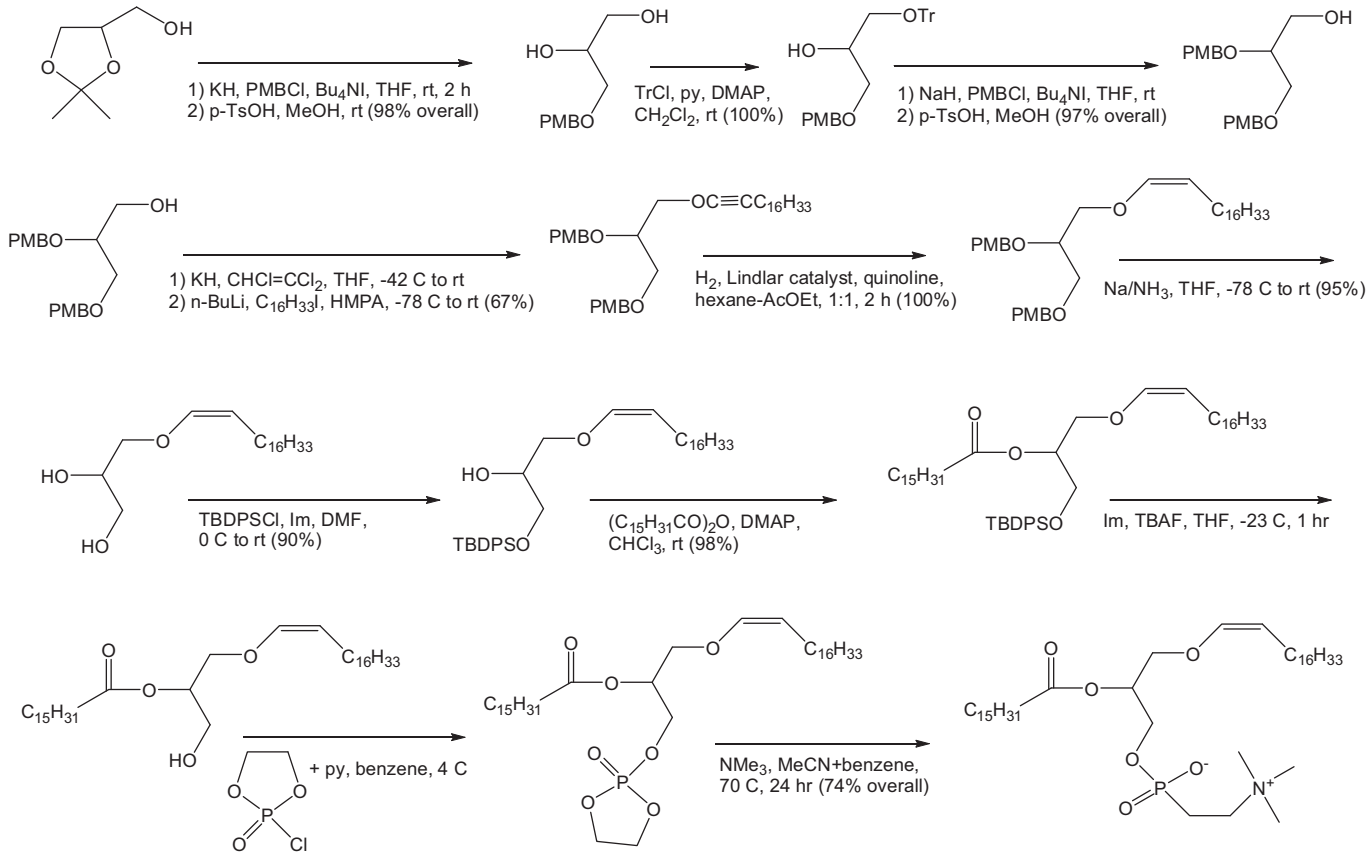


Fig. 4. Total organic synthesis of plasmalogen phosphatidyl choline [38]. Abbreviation: Bu_4NI, tetrabutylammonium iodide; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; HMPA, hexamethylphosphoramide; Im, imidazole; MeCN, acetonitrile; n-BuLi, n-butyllithium; NMe_3, triethylamine; PMBCl, 4-methoxybenzyl chloride; p-TsOH, p-toluenesulfonic acid; py, pyridine; TBAF, tetra-n-butylammonium fluoride; TBDPSCl, tert-butyl(chloro)diphenylsilane; THF, etrahydrofurane; TrCl, trityl chloride;

can be hydrolyzed in both basic and acidic environments, alkenyl chains are stable in basic environments. In contrast, in an acidic environment, traces of acid, such as HCl, formed from chlorinated hydrocarbons, lead to the formation hydrolytic products - aldehydes. Aldehydes can be analyzed in the free form, but far more often, preferably, as their derivatives e.g. dimethyl acetals or cyclic acetals. They can be prepared by acid hydrolysis of intact plasmalogens using BF_3 etherate and alcohol. This reaction produces methyl esters of fatty acids and dimethyl acetal from long chain fatty aldehydes. At present, their separation on capillary columns is not a problem. Dimethyl acetals always eluted ahead of the corresponding fatty acids. If fatty aldehydes do not only have straight chains, aldehydes (not acetals) can either be reduced to alcohols, e.g. sodium aluminium hydride or oxidized by Jones reagent (solution of chromium trioxide in aqueous sulfuric acid) to fatty acids. Fatty acids can be advantageously converted to 3-pyridylcarbonyl esters (formerly called picolinyl esters) or fatty alcohols to nicotinates. Both types of nitrogen derivatives have very well identifiable mass spectra, especially with regard to the identification of the position of double bonds, branching (e.g. iso or anteiso alcohols) or the cyclopropane ring.

Phospholipase C hydrolyzes the polar part of the phospholipid to form alkyl-acyl, alkenyl-acyl and diacyl-glycerols. Their identification is possible by HPLC [34,43], or after derivatization of the free hydroxyl group by GC [44]. All of these methods are time consuming and labor intensive, and result in the loss of information on the molecular species of the respective phospholipid(s). Over the last ten years, along with the development of analytical techniques, mainly HPLC and mass spectrometry, two techniques have been widely used, i.e., shotgun analysis and LC-MS, both with electrospray ionization. Research describing the separation of alkenyl-acyl and diacylphospholipids on a diol-stationary phase column was published [45] and several other publications described separation on an HPLC column with a diol-bound stationary phase [36,46,47]. A highly complex mixture of up to five solvents was used as the mobile phase, which also contained a buffer composed of both acetic and formic acids and a base (ammonia, ammonium acetate, trimethylamine, etc.). Unfortunately, the order of elution of individual classes of phospholipids differed; in contrast, the elution of plasmalogens always preceded the elution of the corresponding diacyl phospholipid.

A known problem in lipidomic analysis is the resolution of isobaric molecular species of phospholipids, even when using high-resolution MS. An example is the analysis of 1-O-alkyl (pl**a**smanyl) and 1-O-alk-1′-enyl (**p**lasmenyl), which cannot be distinguished from each other; see the structural formulae in Fig. 6. This is especially true in LC-MS/MS, which often leads to inaccurate or even incorrect analytical results. In addition, as noted above, this problem is exacerbated by the limited supply of commercially available plasmanyl and plasmenyl standards. Therefore, a method using LC-MS/MS was developed that could separate individual plasmalogens and identify 1-A-alk-1′-enyl and 1-A-alkyl ether lipids in wild and genetically modified mice [48]. The presence of a vinyl ether group in isobaric molecules has been found to cause a shift in retention time (tR) different from the contribution of other double bonds in hydrocarbon chains, e.g., A-18:1/20:4-PE and P-18:0/20:4-PE (Δ tR = 57 s) and A-16:0/22:5-PE and P-16:0/22:4-PE (Δ tR = 45 s). Therefore, they can be separated by Reversed-Phase-High Performance Liquid Chromatography (RP-HPLC).

A new method for the identification of PEs has been developed and applied, for both diacyl-PEs and alkenyl-acyl PEs [49]. The novelty of this method lies in the finding that Ag^+ ions form adducts, which in

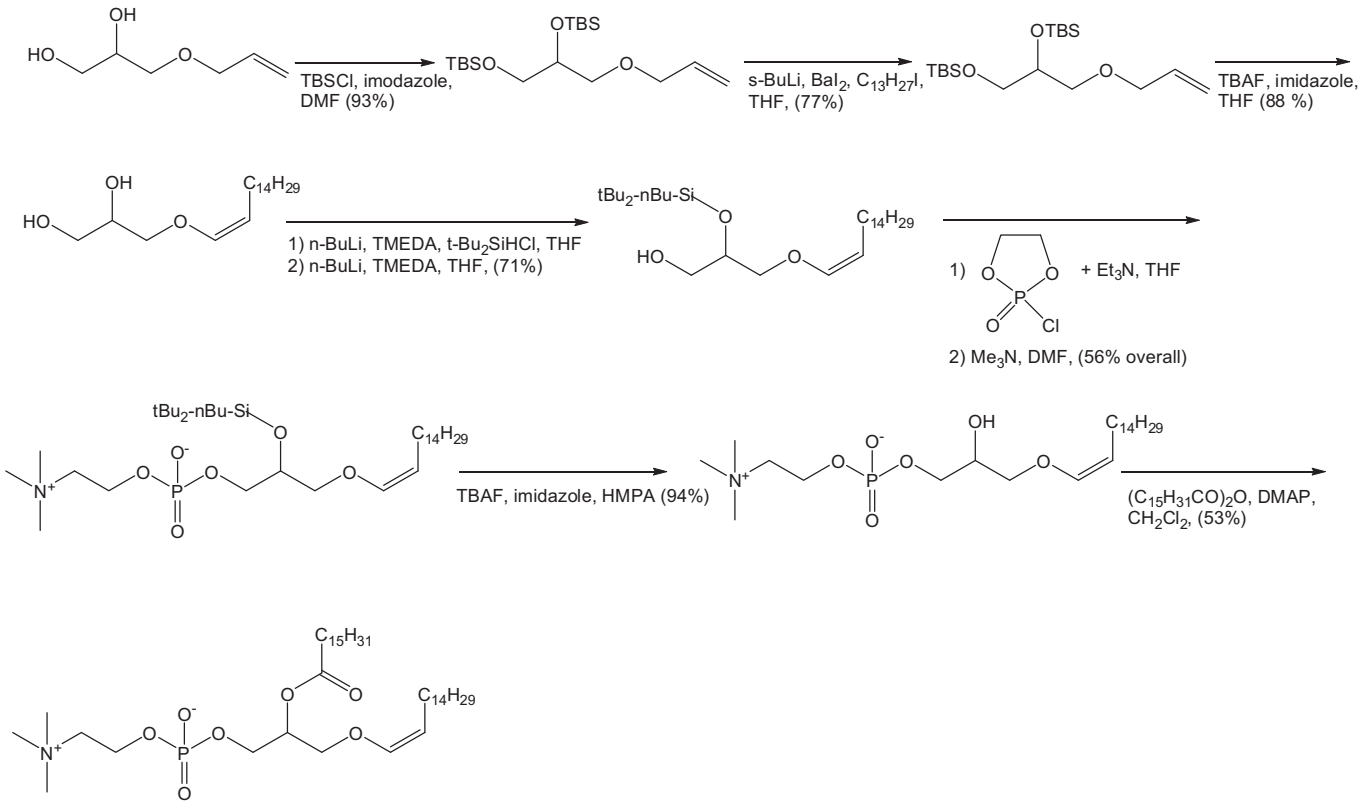
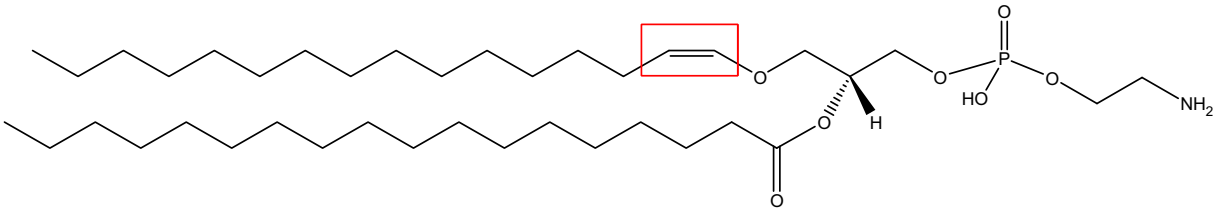
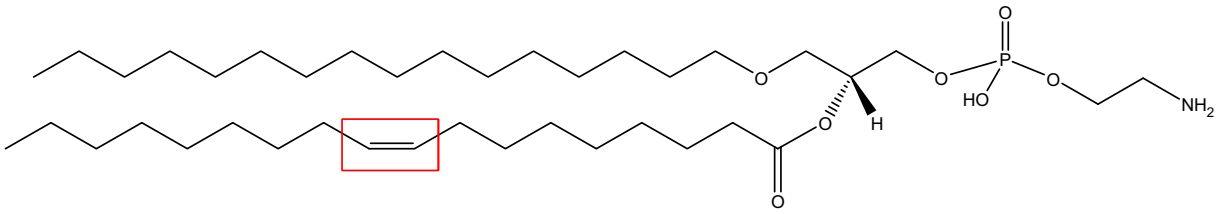


Fig. 5. Total organic synthesis of plasmalogen phosphatidyl choline [40]. Abbreviation: DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; Et_3N, triethylamine; HMPA, hexamethylphosphoramide; n-BuLi, n-butyllithium; s-BuLi, sec-butyllithium; TBAF, tetra-n-butylammonium fluoride; TBSCl, *tert*-butyldimethylsilyl chloride; t-Bu_2SiHCl, di-tert-butylchlorosilane; THF, tetrahydrofurane; TMEDA, tetramethylethylenediamine;



Chemical Formula: $C_{39}H_{78}NO_7P$
Exact Mass: 703.5516



Chemical Formula: $C_{39}H_{78}NO_7P$
Exact Mass: 703.5516

Fig. 6. The structure of 1-O-alkyl (plasmanyl) and 1-O-alk-1′-enyl (plasmenyl) PE having the same MW.

collision-induced dissociation (CID) show a neutral loss of 141 Da (head group) at higher collision energies (~ 50 V). A distinction can be made between diacyl- and alkenyl-acyl-PEs based on the formation of these adducts. Diacyl-PEs form both protonated and Ag^+ adducted forms, whereas P-PEs form only Ag^+ adducted forms. This new approach thus makes it possible to use this phenomenon to identify plasmalogens in a complex lipid mixture.

4.3. Analysis of phospholipids by HILIC and 2D chromatography

Phospholipids are amphiphilic molecules and therefore their separation by HILIC can be carried out directly. This method was used about ten years ago, initially with quite controversial results. The elution order of the individual phospholipid classes varied, e.g., Schwalbe-Herrmann et al. [50] described the elution order of PG, PE and PC, while Kamleh

et al. [51] vice versa, i.e., PC, PS and PG and Scherer et al. [52] published the order PG and PA. From other publications, it is clear that in the separation of plasmalogens and diacyl-phospholipids, plasmenyl-PE always eluted before diacyl-PE.

Unfortunately, HILIC was primarily used to divide classes of lipids isolated from mammalian organs, including humans. The identification of many dozens of molecular species from different classes of lipids in pig organs (brain, heart, kidney, liver, lung, spinal cord, spleen, and stomach) was described [53]. Using HILIC, classes of lipids were separated and further identified by RP-HPLC. In the case of phosphatidylethanolamines, both diacyl-, alkyl-acyl-, and alkenyl-acyl-PEs were identified. Similar results were obtained with phospholipids from mouse brain [54]. Using HILIC in the first dimension and RP-HPLC in the second, with identification using high-resolution mass spectrometers, molecular species were identified, making it possible to demonstrate changes in mice with high and low anxiety-related behaviors. An analysis of lipids from a wide variety of sources has been published, i.e., vascular plants (kale leaves and corn roots), rat brain and soil microbes [55]. The use of all the advantages of 2D technology (HILIC and RP-HPLC) has led to the identification of many molecular species. This approach has made it possible to discover new biomarkers and to study lipid metabolism in a wide variety of biological samples.

In addition, the separation and identification alkenyl-acyl and diacyl phospholipids or lysophospholipids were carried out using HILIC-based UHPLC [56]. Similarly, the application note described 2D (HILIC and RP-HPLC) analysis of blood plasma and induced sputum samples, in which plasmalogens form a substantial part of the lipids [57].

Molecular species of seventeen P-PEs and four P-PCs in chicken breast meat were identified by 2D-HPLC [58]. The main approach was the quantification of molecular species and the least amount of sample for analysis was 12 mg. Furthermore, vinyl ether linkages on *sn*-1 are preferentially oxidized over PUFAs have double bonds in phospholipids upon exposure to various free radicals and singlet oxygen groups. Plasmalogens are thought to play a role as sacrificial oxidants instead of PUFAs and other sensitive membrane lipids [59].

The use of LC-MS, i.e. hydrophilic interaction liquid chromatography coupled with mass spectrometry for phospholipidomic profiling of serum samples from patients with multiple sclerosis has been described [60]. In the case of patients with multiple sclerosis, the phospholipidoma profile has a completely different composition than in the control sample, ie healthy patients. In particular, P-PC and P-PE as well as diacyl derivatives of phospholipids, which contain polyunsaturated fatty acids, were present in significantly lower levels in patients with multiple sclerosis and in patients either in remission or relapse. This demonstrated that serum phospholipidomes are different and can be used as biomarkers for the clinical assessment of multiple sclerosis.

Kadokawa et al. [61] suggest that there is an age difference in the quality, quantity and ability of the bovine brain to use ethanolamine plasmalogens to stimulate bovine gonadotrophs. The brains of about 26 month old heifers and about 90 month old cows were compared. Molecular species of P-PE were compared using 2D LC-MS and 20 molecular species of P-PE were identified. The authors thus assume that P-PE in the old-brain may be associated with age-related infertility.

4.4. Analysis of different types of phospholipids by mass spectrometry

Mass spectrometry is the most important method for structural characterization of all classes of glycerolipids. In the last 10 years, soft ionization techniques have been used, the principle of which is formation of ions in the gas phase without extensive fragmentation. For less polar lipids, such as triacylglycerols or alkenyl diacylglycerols, atmospheric pressure chemical ionization (APCI) can be used. Two techniques are mainly used for the analysis of polar lipids; matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI). Cell lipids are complex mixtures that differ in fatty acyls or alkyls on a glycerol backbone. Polar groups are then attached at the *sn*-3 position of glycerol. Soft ionization techniques usually generate ions such as $[M + H]^+$, $[M + NH_4]^+$ or $[M +$ alkali metal ions (Li, Na or K)$]^+$ in positive ion mode and ions $[M\text{-}H]^-$ in negative mode.

Two analytical methods have been described. The first uses a combination of MS and LC. The second method uses direct injection into the MS and is often called a shotgun method. In the case of HPLC-MS coupling and using different columns, e.g., HILIC or reverse phase, lipids are divided on the basis of a polar group, sometimes also called a head group, e.g., for phospholipids they are usually phosphoric acid derivatives. In the case of reverse phase, the polarity of hydrocarbon chains has the greatest influence on the order of elution from the column, e.g., chain length, number and configuration of double bonds, chain branching, etc. In the case of shotgun analysis, the sample is injected directly into the mass spectrometer and usually further analyzed by high-resolution tandem MS. High-resolution makes it possible (at least for commonly identified molecular species of lipids) to determine the summary formula of the compound(s). Subsequent analysis by tandem MS could confirm the structure of the molecule, e.g., in the case of phospholipids also the binding of individual acyls in the *sn*-1 and/or *sn*-2 positions on the glycerol backbone. The original publication identifying plasmalogens was published by Hsu and Turk [62], where, using ESI-MS or ESI-MS/MS, they identified intact plasmalogens as lithiated adducts ($[M + Li]^+$). The tandem of MS of molecular ions and their cleavage to form characteristic fragments enabled the identification of individual molecular species. In the presence of lithium salts, molecular species of plasmalogens form $[M + Li]^+$ ions producing ion spectra that contain diagnostic fragments for both the polar head group and the fatty acyl (alkyls, alkenyls) groups. Table 3 provides illustrative cases of both LC-MS and shotgun analysis, which were used to separate and identify plasmalogens.

LC-ESI-MS^2 was used to analyze phospholipids from three species of anaerobic beer-spoilage bacteria of the genus *Pectinatus* [7]. Analysis of total lipids by HILIC separated diacyl and alkenyl-acyl phospholipids. Plasmalogens were then analyzed by ESI-MS^2, and more than 220 molecular species of four classes of phospholipids, i.e. P-PC, P-PE, P-PG and P-PS, were identified. The method showed excellent reproducibility, high sensitivity (from 1 μmol/mL) and dynamic range (about 4 orders of magnitude). It was found that all classes of phospholipid plasmalogens were eluted in the first step followed by the corresponding diacyl phospholipids separated almost at the baseline. Elution can be conveniently performed using an aqueous phase with an ESI-compatible buffer.

Compared to LC-MS lipid analysis, shotgun analysis offers the potential to obtain a mass spectrum containing ions of individual molecular lipid species at a constant solute concentration during direct infusion. Scanning of precursor ions and/or the neutral loss of individual lipid molecular species can then be used to identify and quantify them without the time constraints common with chromatographic elution (HPLC). Because most lipid classes represent combinations of polar head groups of fatty acids or alkyl (alkenyl) chains, each series of scans then determines the identity of the molecular ion. In contrast, shotgun analysis made it possible to determine the contamination of beer e.g. by *Pectinatus* and *Megasphaera* where only 830 cells/mL were present [63]. The whole analysis was based on the presence of a specific plasmalogen (1-(methylene-nonadecyl)-2-pentadecanoyl-*sn*-glycero-3-phosphoethanolamine (c-p-19:0/15:0)), i.e. where the range of detection was 2 pg to 20 ng.

On the other hand, the main advantage of LC-MS over shotgun analysis is that LC-MS or LC-MS/MS can be used for quantitative analysis of different types of lipids, differing mainly in FAs, e.g., unsaturated fatty acids would be separated by HPLC from cyclopropane FAs. This separation and identification of unsaturated- or cyclopropane FAs cannot be performed by tandem MS; see for example the note "indicating that the 17:1 fatty acid may represent a 9,10-methylene hexadecanoic acid or cyclopropane fatty acid rather than an unsaturated fatty acid" in the report by Hsu and Turk [64].

Modern analytical techniques have been used to diagnose markers for the detection of colorectal cancer in the early stages of the disease

Table 3
Sources of plasmalogens, HPLC techniques and analytical conditions used for their separation and identification.

Ref.	Column Type	Mobile Phase; temperature/Infusion Solvent/Matrix	Flow (mL/min)	Ion Source - analyzer	Source Conditions	Scan Type	Mass Range (*m/z*)	Samples Matrix	Notes
[130]	Direct MS analysis	Nitrobenzylalcohol matrix		FAB - MSMA		+mode		Synthetic plasmanyl LPC	
[131]	Direct MS analysis	10 mM $AcONH_4$ in MeOH/$CHCl_3$ (75:25)		ESI^+ – qQq	4.5 kV	TIC; precursor ion scan (184 *m/z*); neutral ion scan (141 *m/z*)	600–1000	Diradyl PC – monitoring of PE *N*-methyltranspherase activity	
[62]	Direct MS analysis	MeOH		ESI - Linear IT	300 °C 4.5 kV	MS^3; MS^4		Diradyl PLs from *Leishmania major* and African trypanosomes	
[79]	Direct MS analysis			ESI - qQq	+700 V -900 V	nano-ESI-MS-MS precursor scanning for *m/z* 153	400–2000	*Trypanosoma brucei* cell culture	
[117]	Lichrosphere 100 diol 2 × 125 mm; 5 μm	A) hexane/*i*-PrOH/HCOOH/ NH_4OH (79:20:1.2:0.04) B) *i*-PrOH /water/ HCOOH / NH_4OH (88:10:1.2:0.04); gradient	0.35	ESI^- - IT	200 °C - 4.5 kV	MS^2; dependent-scan mode		Plasmanyl PLs from sulfate-reducing bacteria *Desulfosarcina variabilis* and *Desulforhabdus amnigenus*	Silica gel column chromatography
[132]	ODS Hypersil 2.1 × 200 mm; 5 μm	MeOH/water/ NH_4OH	0.40	ESI^+ – Q	270 °C - 4 kV	CID	100–1100	Synthetic mixtures of plasmanyl-glycerols	
[133]	Zorbax SB-C8 2.1 × 50 mm; 5 μm	A) MeOH/ACN/ 1 mM $AcONH_4$ (60:20:20) B) 1 mM $AcONH_4$ in EtOH; gradient; 30 °C	0.20	ESI^+ and ESI^- - qTOF	- 4.2 kV +5.5 kV	CID		Pls of PE, PG, CL and *N*-acetylglucosaminyl diradylglycerol of *Clostridium tetani*	
[7]	ZIC-HILIC 2.1 × 250 mm; 3.5 μm	A) ACN/10 mM $HCONH_4$, 0.5 mM LiOH, and 0.5 mM AcOLi in water (80:20) B) 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	TIC; CID	200–1100	Pls of PG, PE, PC, PS from *Pectinatus*	
[90]	Ascentis Si HPLC column, 25 cm × 2.1 mm, 5 μm,	A) chloroform/methanol/ammonium hydroxide (800:195:5) B) chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5) C) chloroform/methanol/water/ ammonium hydroxide (450:450:95:5), gradient	0.3	ESI^- - Q	−4.500 kV	MS^2		PLs biosynthetic pathway in anaerobic *Clostridium novyi*	2D-TLC
[55]	HILIC	3 μm, 100 × 2 mm I.D., particle size: 3 μm, acetonitrile:water (97:3 *v*/v) + 10 mM ammonium acetate and 10 mM ammonium acetate in water, gradient	0.2	ESI^-	3.2 kV	resolution of 70,000 *m/z* or 35,000 *m/z* for MS/MS	200–2000 *m/z*;	kale leaves, corn roots, rat brain, and soil microbes	
[55]	C30	150 × 2 mm I.D., particle size: 2.6 μm, acetonitrile: H2O (60:40 *v*/v) and isopropanol:acetonitrile:water (90:10:1 v/v/v) both containing 10 mM ammonium formate and 0.1% formic acid, gradient	0.2	ESI^- and ESI^+					
[66]	C8	2.0 × 100 mm; methanol: water 85:15 (v: v) containing 0.1 mM sodium acetate or 5 mM ammonium acetate and methanol containing 0.1 mM sodium acetate or 5 mM ammonium acetate, gradient	0.3	ESI	5 kV	MRM, multiple reaction monitoring	product ion (184.0 Da) for diacyl-PE or 392.3 Da for P-PE	human plasma, Alzheimer's disease	P-PCs and P-PE species 0.5–13.6 μM (Limit of Detection (LoD))

(*continued on next page*)

Table 3 (*continued*)

Ref.	Column Type	Mobile Phase; temperature/Infusion Solvent/Matrix	Flow (mL/min)	Ion Source - analyzer	Source Conditions	Scan Type	Mass Range (*m/z*)	Samples Matrix	Notes
[54]	HILIC	150 × 2.1 mm, 2.6 μm, gradient 50 mM ammonium formate-acetone)	0.4	ESI	2.5 kV	resolution of 35,000	100–1000	mouse brain, dorsal hippocampus, ventral and prefrontal cortex	
[54]	C18	2.1 × 50 mm, 1.7 μm, water/acetonitrile (50:50, v/v) and water/acetone (5:95, *v/v*), containing 5 mM ammonium formate	0.4	ESI	2.5 kV	resolution of 35,000	100–1000	mouse brain, dorsal hippocampus, ventral and prefrontal cortex	
[87]	silica normal-phase	150 mm × 4.6 mm, i.d. 3 μm, chloroform and methanol/28% ammonia in water/chloroform (92:7:1, *v/v/v*), gradient	1.5	ELSD Evaporative light scattering detector	–	–	–	tropical tunicates *Eudistoma sp. and Leptoclinides uniorbis*	
[53]	HILIC	Spherisorb Si column (250 × 4.6 mm, 5 μm) acetonitrile and 5 mM aqueous ammonium acetate, gradient	1	ESI^- and ESI^+			50–1000	Brain, heart, kidney, liver, lung, spinal cord, spleen, and stomach of pig	
[53]	Kinetex C18	(150 × 2.1 mm, 2.6 μm), acetonitrile–2-propanol (1:1, *v/v*) and 5 mM aqueous ammonium acetate.	0.3	ESI^- and ESI^+			50–1500	Brain, heart, kidney, liver, lung, spinal cord, spleen, and stomach of pig	
[134]	C18	(4.6 × 250 mm, 5 μm), methanol/0.5 M ammonium acetate (99:1, v/v)	1	electrospray ionization-time of flight mass spectrometry (ESI-TOF MS)	2 kV			marine invertebrates, *Crassostrea gigas, Patinopecten yessoensis, Todarodes pacificus, Paroctopus dofleini, Marsupenaeus japonicas*, and *Halocynthia roretzi*	
[89]	Ascentis Si HPLC	5 μm, 25 cm × 2.1 mm chloroform/methanol/aqueous ammonium hydroxide (800:195:5, v/v/v/v), chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, v/v/v/v.), chloroform/methanol/water/aqueous ammonium hydroxide (450:450:95:5, v/v/v/v), gradient	0.3	ESI triple TOF	ESI^+ 5.5 kV ESI^- -4.5 kV	resolution >25,000	MS at *m/z* 200–2000 MS/MS at *m/z* 50–2000	*Clostridium cadaveris*, *C. fallax*	diradylglycerol, monohexosyldiradylglycerol, P-PG as diacyls and plasmalogens, CL as tetraacyl, monoalkenyl-triacyl, and dialkenyl-diacyl forms
[86]	Cosmosil HILIC column	150 mm × 4.6 mm; 3 μm, acetonitrile, 60 mM ammonium formate and 0.1% formic acid aqueous solution, gradient	0.6	ESI-	−4.5 kV		600–1000	six edible shellfishes - mussel (*Mytilus edulis*), abalone (*Haliotis rubra*), scallop (*Placopecta magellanicus*), oyster (*Ostrea gigas*), clam (*Venerupis variegata*), and razor clam (*Sinonovacula constricta*)	
[135]	direct inlet		2.5 μL/min	ESI MS/MS	5 kV		100–1000	brain and heart of rats	MS/MS with alkali metals (Li, Na, K)
[135]	C18	0.5 × 150 mm, water containing 0.2 mM sodium acetate and methanol containing 0.2 mM sodium acetate, gradient	0.1		5.5 kV				
[92]	direct inlet	–	5–10 μL/min	QTOF ESI/MS	ESI+ 5.5, ESI- -4.2 kV	MS^2		strain of *C. tetani*	P-PE, P-PG, tetra-acyl, tri-acyl-alkenyl, and diacyl-dialkenyl-CLs were present
[92]	Zorbax SB-C8	5 μm, 2.1 × 50 mm, methanol/acetonitrile/aqueous 1 mM ammonium acetate (60/20/20, v/*v*/v) and 1 mM ammonium acetate	0.2	QTOF-MS as above					10% of the LC flow to ESI MS
[58]	NP-HPLC	YMC-Pack PVA-Sil (250 mm × 4.6 mm, 5 μm) n-hexane, MTBE,	1	variable wavelength detectors (VWD) 210 nm				soybean lecithin and chicken breast meat	

(*continued on next page*)

Table 3 (*continued*)

Ref.	Column Type	Mobile Phase; temperature/Infusion Solvent/Matrix	Flow (mL/min)	Ion Source - analyzer	Source Conditions	Scan Type	Mass Range (*m/z*)	Samples Matrix	Notes
[58]	RP-HPLC C18;	250 mm × 4.6 mm, 3 μm, acetonitrile/ MeOH/ 20 mM ammonium acetate (25/ 68.5/6.5, v/v/v %)	1	ESI^+	5 kV	MS^2	300–2000	soybean lecithin and chicken breast meat	split into charged aerosol detector (CAD) and ESI-MS (4/1)
[48]	RP-HPLC C8	2.7 mm 2.1x100mm 4/6 H_2O/ ammonium format, 0.2% formic acid and 9/1 isopropanol/acetonitrile, 10 mM ammonium formate, 0.2% formic acid	0.4–0.6 gradient	ESI^-	3.8 kV	CID	460–1650	deficient enzyme mice	
[125]	NP-HPLC	5 μm, 25 cm × 2.1 mm	0.3	ESI^-	4.5 kV	MS^2		*Romboutsia* sp., *R. lituseburensis, R. ilealis*	no alk-1-enyl ether lipids (plasmalogens) were present in Romboutsia but *C. difficile* is closely related (>95% sequence identity).
[91]	HILIC	100 × 4.6 mm, 2.6 μm, 10 mM ammonium formate in water containing 0.5% formic acid and isopropanol/ acetonitrile (5:2) containing 10 mM ammonium formate and 0.5% formic acid, gradient	0.3	ESI^+ (PA, PG) neutral loss scan at 172 Da, ESI^- (CL) precursor ion scan at 153 Da				*Bifidobacterium longum, B. animalis*	
[136]	HILIC	2.7 μm, 2.1 mm × 150 mm, water and acetonitrile/water (95/5), both contained 0.1% formic acid and 0–10 mmol/L ammonium formate, gradient	0.3	ESI^- and ESI^+	3.5 kV	MS^2 100–800 MS^3 100–700	100–1000	human plasma (over 1000 residents)	
[90]	NP-HPLC (Si)	5 μm, 25 cm × 2.1 mm, chloroform/ methanol/aqueous ammonium hydroxide (800:195:5, by vol.), chloroform/methanol/water/aqueous ammonium hydroxide (600:340:50:5, by vol.) and of chloroform/methanol/ water/aqueous ammonium hydroxide (450:450:95:5, by vol.).	0.3	ESI^-, ~10% of the LC flow to the ESI	4.5 kV	MS^2			
[49]	–	–	direct injection 7 μL/min	ESI^+	4.5 kV	CID of Ag(I)-adduct of p-PE and neutral loss scan(141 Da)		standards 18:0/22:6-P-PE, 18:0/ 20:4-P-PE, 18:0/18:1-P-PE, PE (soybean)	
[137]	HILIC	Spherisorb Si column (250 × 4.6 mm, 5 μm), acetonitrile and 5 mM aqueous ammonium acetate, gradient	1	ESI^- and ESI^+			50–1000	human breast tumor tissues and surrounding normal tissues	
[57]	HILIC	RX-SIL, 1.0 × 150 mm, 3.5 μm, 0.1% formic acid in acetonitrile and 20 mM ammonium formate in acetonitrile/ methanol/water, 50/20/30, *v/v/v*	40 μL/min	ESI^- and ESI^+	3,5 kV	200–1700		lung sputum and human plasma	
[57]	RPLC	C18 RRHD, 2.1 × 100 mm, 1.8 μm, 20 mM ammonium formate/0.1% formic acid in methanol/water, 10/90, *v/v* and 20 mM ammonium formate/0.1% formic acid in acetonitrile/methanol/ isopropanol, 20/30/50, v/v/v	0.6	ESI^- and ESI^+				lung sputum and human plasma	resolution 10,000 for *m/z* 1000
[80]	Inertsil SIL-100A	2.1 × 100 mm, 3 μm; acetonitrile–methanol–1 M aqueous ammonium formate (78:20:2,by vol) and acetonitrile–methanol–1 M aqueous	0.2		4.5 kV and + 4.8 kV	Precursor ion scanning at 196 Da	100–900	brown algae, blue mussel, scallop, salmon, cattle, pig, chicken, etc.	

(*continued on next page*)

Table 3 (*continued*)

Ref.	Column Type	Mobile Phase; temperature/Infusion Solvent/Matrix	Flow (mL/min)	Ion Source - analyzer	Source Conditions	Scan Type	Mass Range (*m/z*)	Samples Matrix	Notes
		ammonium formate (49:49:2, by vol), gradient							
[83]	Nucleosil OH column	4.6 mm × 250 mm, 5 μm, n-hexane/isopropanol/formic acid/ammonia water (70:30:1:0.08, *v/v*) and isopropanol/water/formic acid/ammonia water (100:13:1:0.2, v/v), gradient	0.5	Negative MS^2	3.5 kV	200–1000 for MS (resolution of 70,000) and 50–900 for MS/MS (resolution of 17,500)		mussel *Mytilus edulis* and bovine brain	
[81]	Hypersil GOLD C8	1.9 μm, 50 mm 2.1 mm, methanol/water 5:1 v/v (with 10 mM ammonium acetate) and methanol, gradient	0.2	ESI^- and ESI^+	3 kV	600–900 (resolving power 60,000)	MS2 and MS3	beef, pork, lamb, chicken, tuna, salmon, shrimp, octopus, etc.	

[65]. Based on the ultra-high performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UHPLC-Q-TOF MS) and ultra high-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UHPLC-QQQ MS) techniques, several hundred molecular species of lipids were detected, and by multivariate data analysis, it was found that identification of some plasmalogens, i.e. P-PEs and A-PEs (alkyl-acyl-PE) differentiated between healthy patients and those having early stage cancer.

The addition of Na^+ ions increased the sensitivity of the assay to the order of fMs (femtomol, i.e. 10^{-15} M) [66]. The recoveries of plasmalogens, both P-PEs and P-PCs from human plasma, were almost quantitative and no matrix effect was observed. These were mainly 18:0/18:1, 18:0/20:4, 18:0/22:6 molecular species, where the limit of detection ranged from dozens of femtomole to nanomol units while the concentration in human plasma was in units of μM.

Almost half a century ago, one of the first analyzes of alkenylacyl, alkylacyl and diacyl acetylglycerols isolated from total bovine brain phospholipids was performed [43]. Total phospholipids were hydrolyzed with phospholipase C and acetylated to give diradylacetylglycerols and then separated by normal phase HPLC. The individual fractions were further separated into molecular species by RP-HPLC. Dozens of molecular species were identified, e.g., 18:1/18:1, 16:0/18:1, 18:0/22:6 (n-3) or 18:0/22:4 (n-6) alkylacyl-acetyl, etc. as major alkenylacyl acetyl derivatives. In essence, many other publications are based on this description of the analysis. One of the first descriptions of plasmenyl lipids in bovine heart was published as early as 1968 [67]. The authors used a series of reactions, including hydrolysis of phospholipase A, phospholipase C, acid hydrolysis with HCl, reduction of aldehydes to alcohol by $LiAlH_4$, and detection of methyl esters and alkyl acetates by GLC (Table 4). In principle, it was a repetition of a previously used methodology, only using more modern techniques such as HPLC instead of TLC [34]. After separation of total phospholipids from mammalian tissues (human, mouse, and rat brains, rat liver) by HPLC, the polar heads were hydrolysed by phospholipase C and the resulting diradylglycerols were separated by RP-HPLC after acetylation. Fatty acids and aldehydes of acetyl diradylglycerols were converted to FAMEs and dimethyl acetals, and were analyzed by capillary GLC.

Multidimensional mass spectrometry-based shotgun lipidomics were analyzed for lipidic extracts from mouse brain and heart [68]. Using tandem MS of neutral loss scanning and precursor ion scanning, molecular species of diacylglycerol and vinyl ether acyl glycerols (obtained by phospholipase C) were identified as adducts with Li^+, Na^+ and NH_4^+.

It is also possible to separate lipids containing isobaric FAs, e.g. straight chain FAs, isoFAs and anteiso-FAs such as triacylglycerols [69], which cannot be distinguished by MS or tandem MS. In addition, HPLC can be used to separate molecular types of lipids that contain positional isomers of fatty acids, such as oleic-vaccenic [70], and α- and γ-linolenic [71].

Lipidomic analysis of chicken egg yolk, using high-resolution mass spectrometry, was described by Wood et al. [72]. Dozens of molecular species of diacyl-PC and/or PE, plasmenyl-PC and/or PE, and plasmanyl-PE have been identified. For example, plasmenyl-PC (34:1, 36:1), plasmenyl-PE (36:2 and 34:1), and plasmanyl-PE (34:0) were predominant.

The effect of water temperature on the plasmalogen content of freshwater fish in the brain of carp (*Cyprinus carpio*) in summer and winter has been investigated [73]. Lipids were separated by TLC and hydrolysis by phospholipase C to form alkenylacyl, alkylacyl and diacyl fractions, which were then acetylated and again separated by TLC. Diradyl-acetyl glycerols were first transmethylated and fatty acid methyl esters were identified by GLC. Molecular species of the phosphatidylethanolamine subclasses of carp brain were also analyzed by RP-HPLC and identified in comparison with commercially available standards. It was found, for example, that in phosphatidylethanolamine subclasses the ratio of PUFAs (n-6 to n-3) decreased in summer. In contrast, the proportion of myristic acid in P-PE was almost three times higher in summer than in winter.

Table 4
Composition (wt%) of aliphatic moieties of alkylacyl-, alkenylacyl- and diacyl-forms of phosphatidylethanolamine of bovine heart muscle [67].

Chain length, number and position* of double bonds	Ethanolamine phosphatides		
	Diacyl	Alk-1′-enyl-acyl	Alkyl-acyl
16:0**	0,5	1,5	5,6
16:1	tr	tr	1,1
18:0	2,7	4,4	5,6
18:1	3,2	3,7	8,8
18:2	13,0	34,3	17,6
18:3(n-3)	tr	tr	0,9
20:3(n-6)	2,4	6,6	6,0
20:4(n-6)	73,0	42,2	27,3
20:5(n-3)	3,0	1,3	5,9
22:4(n-6)	1,0	2,9	4,4
22:5(n-3)	1,2	3,1	16,8

* Major isomer.
** Traces of 14:0, 15:0, 17:0. 19:0, 20:0, 20:1 and 22:6 were also present in all lipid classes listed.

4.5. Phosphorus-31 nuclear magnetic resonance spectroscopy (^{31}P-NMR)

^{31}P-NMR spectroscopy is an analytical technique that uses nuclear magnetic resonance to study chemical compounds that contain phosphorus. Phosphorus is commonly found in phospholipids, so it is useful to routinely measure ^{31}P-NMR spectra (Table 5). The use of ^{31}P-NMR is one of the more routine NMR techniques. The only stable isotope of phosphorus is ^{31}P with a relatively high gyromagnetic ratio. The ^{31}P nucleus also has a spin of 1/2, so the spectra are relatively easy to interpret. Chemical shifts refer to 85% phosphoric acid, which is assigned a chemical shift of 0 ppm. ^{31}P-NMR is much less sensitive than ^{1}H NMR but more sensitive than ^{13}C NMR. Due to the inconsistent nuclear Overhauser effect, integration is usually not used. The spectra are most often recorded with decoupled protons. ^{31}P-NMR spectroscopy is useful for determining structures of phosphorus-containing compounds because these signals are well resolved and often occur at characteristic frequencies. The usual range of chemical shifts is from about 250 to −250 ppm, which is a much broader spectrum than is typical for ^{1}H NMR. ^{31}P NMR spectroscopy is widely used to study phospholipid bilayers and biological membranes under native conditions. Analysis of ^{31}P-NMR spectra of lipids could provide a wide range of information on lipid bilayer packaging, phase transitions (gel phase, physiological liquid crystal phase, ripple phases, non-bilayer phases), orientation/dynamics of lipid head group, and elastic properties of a lipid bilayer.

Total lipids of the fresh water sponge *Eunapius fragilis* were analyzed by ^{31}P NMR. Eighteen classes of lipids were identified, including plasmalogen lipids, e.g. lysoethanolamine plasmalogen, ethanolamine plasmalogen, phosphatidylglycerol plasmalogen. In all cases, diacyl- and alkenyl-acyl-phospholipids could be distinguished from each other, e.g. the chemical shift of P-PE was 0.06 ppm, while PE was 0.02 ppm. The concentration of phospholipid classes varied depending on the site of collection (lake, lagoon, or river) and reached, for example, a value of about 20 mol% for P-PE, making this phospholipid one of the major lipids [74].

Similarly, ^{31}P NMR spectroscopy was used to analyze phospholipids in bovine and ovine milk fats. Again, many classes of lipids were identified, including P-PE and PE. The differences in chemical shifts were found between alkenyl-acyl- and diacyl-phospholipids. However, it should be noted that internal references must always be taken into account when identifying chemical shifts of phospholipid classes. In this case, PC was used, which has a difference of 0.37 ppm based on 85% orthophosphoric acid or trimethylphosphine (1.97 ppm with respect to 85% orthophosphoric acid). The content of P-PE in ovine and bovine milks was 6.5 and 4.6 mol%, respectively, while PE were 27.5 and 25.8 mol% of total phospholipids, respectively [75].

Table 5
Phospholipid ^{31}P NMR chemical shifts [138].

Phospholipid	Chemical shift (ppm)
Trimethyl phosphate (quantitative and qualitative reference)	1.71
Lysophosphatidylglycerol	1.10
Lysophosphatidic acid	0.91
Glycerol plasmalogen	0.55
Phosphatidylglycerol	0.52
Lysoethanolamine plasmalogen	0.47
Lysophosphatidylethanolamine	0.43
Phosphatidic acid	0.29
Alkylacylphosphatidylethanolaminec	0.22
Diphosphatidylglycerol (cardiolipin)	0.18
Ethanolamine sphingomyelinc	0.16
Dihydrosphingomyelin	0.13
Ethanolamine plasmalogen	0.07
Phosphatidylethanolamine	0.03
Lysophosphatidylinositol	0.00
Phosphatidylserine	−0.06
Di-oleyl phosphate	−0.08
Sphingomyelin	−0.09
Dimethylphosphatidylethanolamine	−0.18
Lysophosphatidylcholine	−0.28
Phosphatidylinositol	−0.37
Alkylacylphosphatidylcholine	−0.77
Phosphatidylcholine	−0.84

To analyze the phospholipid composition of mammalian pulmonary surfactant, two different sources, i.e. lung of an adult cow and lamb fetal pulmonary liquid, were used for high resolution ^{31}P NMR spectroscopy. It was shown that P-PC exists as a major secondary component (about 4 mol%) in mammalian pulmonary surfactant. The practical detection limit for these samples was about 25–50 pg of phospholipid. Furthermore, the effect of the plasmenyl bond in the *sn*-1 position of PC on phase properties, structure, and spreading dynamics of model phospholipid monolayers at the air-water interface was demonstrated [76].

Phospholipids from eyelids of rabbits were analyzed by ^{31}P NMR. The meibomian gland has been found to secrete lipids in the form of meibomian oil. This oil is a mixture of many phospholipids, including P-PE and PE. ^{31}P NMR chemical shifts were 0.03 ppm for PE and 0.07 ppm for P-PE (reported relative to 85% phosphoric acid), so it was not a problem to quantify them. For example, the proportions of P-PE and PE in meibomian oil were 8.5 and 18.0 mol%, respectively [77].

Using ^{31}P NMR, the physical properties of the wild-type anaerobic Gram-negative bacterium Megasphaera elsdenii were compared with a mutant of the same bacterium. Unlike the wild-type (plasmalogen content more than 75%), this mutant contained less than 5% plasmalogens. It was found that the formation of the hexagonal phase in the wild-type, occurred at a temperature above 30 °C, and that phospholipids in the mutant strain (with minimal plasmalogens) produced a more stable lamellar phase at all temperatures examined. Furthermore, increased production of unsaturated and cyclopropane chains was found, where the content reached up to about 95% of all acyls in the mutant strain [78].

5. Analysis of ether phospholipids in lower animals

More than 500 molecular species of phospholipids in *Trypanosoma brucei* (*Trypanosoma* is a endoparasitic protozoa that causes sleeping sickness), including dozens of plasmanyl and plasmenyl phospholipids were identified [79].

Recently, much attention has been paid to the presence of plasmalogens in the diet. Yamashita et al. [80] analyzed brown algae, mussels, other invertebrates (*Todarodes*, *Halocynthia*), fish (salmon and amberjack), chickens and mammals (cow, pig). The quantitative analysis of ethanolamine plasmalogen species in foodstuffs was performed for four molecular species (18:0/18:1-P-PE, 18:0/20:4-P-PE, 18:0/20:5-P-PE, 18:0/22:6-P-PE). Of the foods examined, the highest

levels of 18:0/20:5-P-PE, 18:0/22:6-P-PE in ethanolamine glycerophospholipid was detected. Individual molecular species were identified by precursor ion scanning for *m/z* 196 in negative ion mode ESI.

Another review on foodstuff samples was published [81]. The samples of beef, pork and lamb meat, chicken breast, fish (tuna, salmon, flatfish, etc.) and invertebrates (octopus, squid, shrimp, etc.) were examined for P-PE and P-PCs. The following molecular species were identified (16:0/18:1-, 16:0/18:2-, 16:0/20:5-P-PE and P-PC) in quantities from one up to several hundred nM/g wet weight.

Lipid content was analyzed in two marine invertebrates (marine bryozoan species - *Berenicea meandrina* and *Dendrobeania flustroides*) [82]. A total of nine aldehydes were identified, indicating the presence of plasmalogens. These aldehydes were from C16 to C19, both branched saturated (DMA i-17:0 or DMA i-18:0) and unsaturated (DMA 19:1). Based on the FA markers present, it was concluded that some lipids were derived from food, e.g. microalgae, protozoans and detritus (probably sponge biofouling based on the presence of 5,9,19–26:3 and 5,9,19–28:3, typical of demospongic acids).

Two plasmalogens were isolated from *Mytilus edulis* mussels and bovine brain [83]. At over 90% purity, the majority were 18:0/20:5- and 18:0/22:6-P-PE from mussels and 16:0/22:6- and A-16:0/20:5-PC from bovine brain. Neurotrophic effects of these plasmalogens were studied in an NGF-induced PC12 cell model.

The content of alkenyl, alkyl and diacyl subclasses of PE and PC were examined in more than thirty invertebrates, etc., arthropods (Arthropoda), annelids (Annelida), mollusks (Mollusca), flatworms (Platyhelminthes) [84]. While plasmenyl phospholipids were only present in trace amounts in many species, alkylacyl and diacyl phospholipids were present in all invertebrates studied. In *Marphysa sanguinea,* which is used as bait in commercial and recreational fishing, almost 90% of total PE comprised plasmenyl PE . Six species of sea cucumbers were analyzed by normal phase liquid chromatography (NPLC) connected to ESI tandem MS [85]. Nearly 300 molecular species of seven classes of phospholipids (PA, PG, PC, PE, PS, PI, and phosphonoethanolamine) were identified. The different classes of phospholipids and the isobaric ether-PL and diacyl-PL molecules in the same class of phospholipids were separated. Sea cucumber phospholipids are therefore promising additives for value-added food, especially for their high content of arachidonic and eicosapentaenoic acids.

In marine invertebrates that are commonly consumed, i.e., in six mollusks (mussel (*Mytilus edulis*), abalone (*Haliotis rubra*), scallop (*Placopecta magellanicus*), oyster (*Ostrea gigas*), clam (*Venerupis variegata*), and razor clam (*Sinonovacula constricta*)) plasmalogens were identified [86]. Nineteen molecular species were identified by LC/MS, of which nine were P-PCs, seven were P-PEs and three were P-PSs. The majority were 18:0/20:5-P-PE, 16:0/22:2-P-PE, and 18:0/22:2-P-PE. The levels of total plasmalogens ranged in the lower tens of μg per mg of organism.

In two marine invertebrates - tunicates (*Eudistoma* sp. and *Leptoclinides uniorbis*), lipids and plasmalogens, among others, were identified [87]. A series of eight C16 to C18 aldehydes were identified as dimethylacetals at levels of 5.0% in *Eudistoma* sp. and 14.2% in L. *uniorbis*.

Three subclasses, i.e. alkenylacyl (plasmalogen), diacyl, and alkylacyl of phosphatidylethanolamines, were enzymatically hydrolyzed (phospholipase C from *Bacillus cereus*), acetylated and analyzed [88]. This method made it possible to determine plasmalogens down to levels of 200 ng. Marine invertebrates of classes containing from 19 to 504 μM plasmalogens/100 g wet weight were sampled (Table 5). Table 6 showed presence of plasmenyl and plasmanyl lipids in marine invertebrates.

6. Analysis of bacterial plasmalogens

In two anaerobic bacteria *Clostridium fallax* and *C. cadaveris*, diradylglycerol (DRG), monohexosyldiradylglycerol (MHDRG), monohexosyl monoacylglycerol (MHMAG), phosphatidylglycerol (PG), and P-PE forms were analyzed and identified by TLC and NPLC-MS [89]. Glycerol acetal of plasmenylethanolamine and ethanolamine-phosphate modified diacylglycerol were also present in the bacteria (Fig. 7).

One of the first studies of the difference between plasmalogen biosynthesis in mammals and bacteria was presented in [90]. The authors showed that in the anaerobic bacterium *Clostridium novyi,* the predominant phospholipids were PE and CL, with other minor lipids included phosphatidylglycerol (PG), lysyl-PG, alanyl-PG, phosphatidic acid (PA), CDP-diacylglycerol, phosphatidylserine (PS), and phosphatidylthreonine (PT). Molecular species containing one (PE) or two (CL) alkenyl chains have been identified in both PE and CL. In contrast, in PA, CDP-diacyl glycerol and PS, only diacyl phospholipids were almost exclusively present [30]. It is also clear that in bacteria, plasmalogens are biosynthesized in one of the last steps of the biosynthetic pathway. In contrast, mammalian plasmalogen biosynthesis occurs at the beginning of the biosynthetic pathway (see Fig. 2).

Plasmalogens (of cardiolipin, phosphatidylglycerol and phosphatidic acid) have been found in intestinal microflora, especially *Bifidobacterium longum*, an important probiotic *Bifidobacterium* [91]. In *Bifidobacterium animalis* subsp. *lactic*, plasmalogens have been identified that are a major component of lipids [58].

The polar lipids of the anaerobic bacterium *Clostridium tetani*, the causative agent of tetanus, were examined [92]. Several strains were analyzed and plasmalogens were identified for PE, PG, CL and *N*-acetylglucosaminyl diradylglycerol. Plasmenyl-PA and P-PS have also been identified in trace amounts, again supporting the hypothesis that in anaerobic bacteria, plasmalogens are biosynthesized at the end of the biosynthetic pathway. It should also be noted that the presence of a gene encoding the relevant enzyme does not automatically mean that the relevant metabolite will be present in the cell.

Chlamydia trachomatis is an obligate intracellular pathogen causing vision loss and is responsible for sexually transmitted diseases. Plasmalogens have been identified in this bacterium, but biosynthesis begins in mammalian peroxisomes [93], demonstrating metabolic cooperation between peroxisomes and bacteria.

7. The transition temperature and fluidity of plasmalogens

Conversion of the ester bond in phospholipids to a vinyl ether bond has a dramatic effect on the orientation of proximal regions of acyl chains with respect to the normal lipid bilayer [94,95]. The vinyl ether bond itself has significant effects on acyl chains, the glycerol backbone and especially on conformational properties and dynamics. These properties have an impact on combined biophysical properties of homogeneous

Table 6
Plasmenyl and plasmanyl lipids in marine invertebrates [134].

marine invertebrate	P-PE	PE	A-PE
Acanthopleura japonica	+	+	+
Anthopleura japonica	+	+	+
Anthopleura midori	+	+	+
Asterias amurensis	+	+	+
Asterina pectinifera	+	+	+
Cellana grata	+	+	+
Crassostrea gaigas	+	+	+
Halocynthia roretzi	+	+	+
Hemicentrotus pulcherrimus	+	+	+
Chlorostoma argyrostoma	+	+	+
Marsupenaeus japonicus	+	+	+
Mytilus galloprovincialis	+	+	+
Nucella heyseana	+	+	+
Paroctopus dofleini	+	+	+
Patinopecten yessoensis	+	+	+
Septifer virgatus	+	+	+
Stichopus japonicus	+	+	+
Strongylocentrotus nudus	+	+	+
Thais bronni	+	+	+
Todarodes pacificus	+	+	+
Tugali gigas	+	+	+

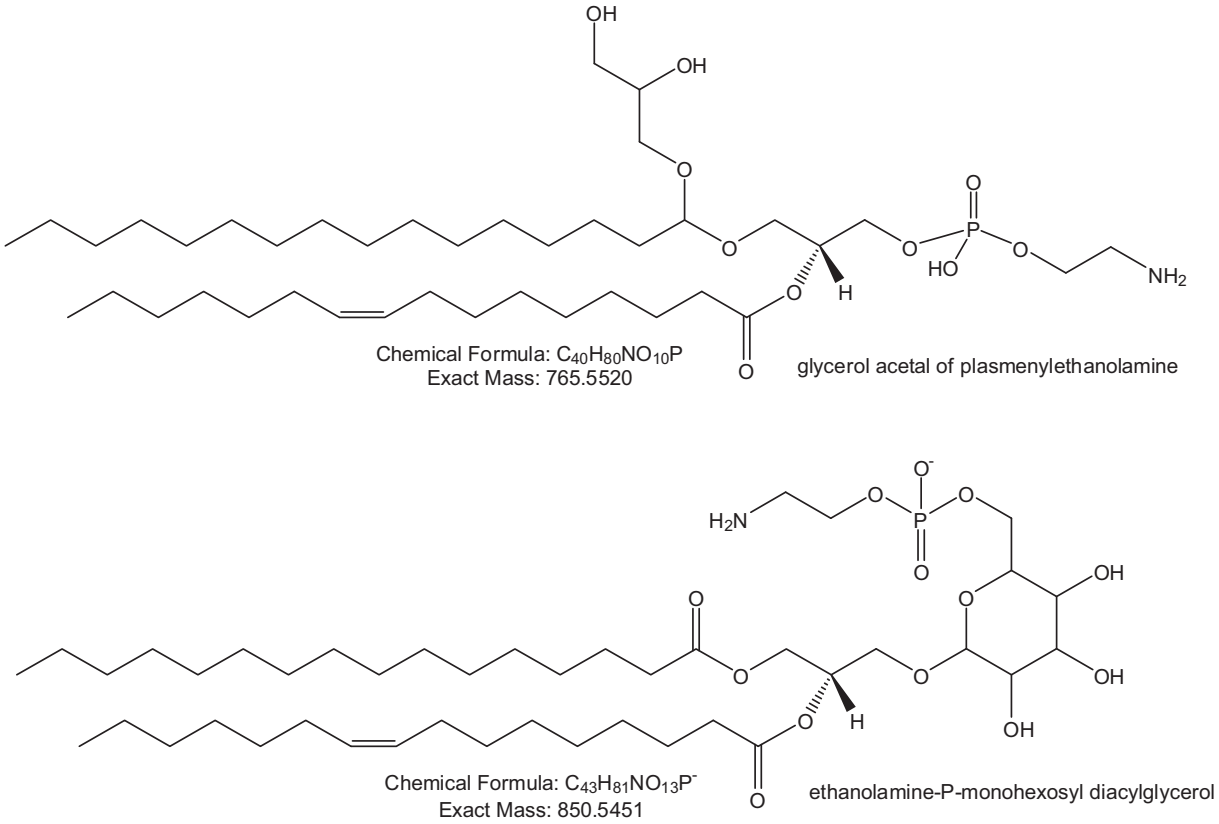


Fig. 7. Some lipids found in various species of *Clostridium* sp.

plasma bilayers and monolayers. In addition, these properties play a key role in determining the physiological role of plasmalogens in heterogeneous lipid membranes.

Orientation of the acyl chain at the *sn*-2 position of plasmalogens is mainly perpendicular to the membrane surface, which leads to an effectively longer aliphatic chain in the plasmalogen analog than in the diacyl analog. In addition, the absence of a carbonyl oxygen at the *sn*-1 position (essentially replaced by a vinyl bond) affects the hydrophilicity of the head group (it is therefore much more lipophilic) and allows stronger intermolecular hydrogen bonds to form between the head groups of lipids. This can increase the lipid content of the glycerol backbone and reduce contacts between water and hydrophobic acyl chains. These properties favor the formation of a non-lamellar structure, which is reflected in the high affinity of ethanolamine plasmalogen to form an inverse hexagonal phase and is a requirement for membrane fusion [96,97].

The gel to liquid crystalline phase transition temperature, as determined by differential scanning calorimetry, was lowest for ethanolamine plasmalogen (26 °C) and was similar for alkylacyl and diacyl analogs (29.5 °C and 30 °C, respectively) [98]. Far greater differences were seen in lamellar structures. Ethanolamine plasmalogen undergoes a lamellar phase transformation to hexagonal (L → H_{II}) at 30 °C, analogous to alkylacyl-PE and diacyl-PE at 53 °C and 69 °C, respectively. Thus, the alkenyl ether bond in position 1 of *sn*-glycerol, which is a structural characteristic of plasmalogens, effectively stabilizes the hexagonal arrangement of H_{II} ethanolamine glycerophospholipids, although it has a relatively small effect on destabilizing the lamellar gel state [98]. Destabilization of the lamellar phase by an alk-1′-enyl chain at the *sn*-1 PE position was described by Boggs et al. [99]. In other words, they play a role in vesicle cleavage and fusion processes, as well as in the formation of other curved membrane architectures. From other measurements, it is clear that the vinyl ether bond increases the stiffness and reduces the packaging of homogeneous and heterogeneous lipid membranes containing plasmalogens. Also, the *sn*-1 chain associated with the vinyl ether slightly increases the thickness of the homogeneous lipid bilayers.

The fluidity and phospholipid content of wild-type *Megasphaera elsdenii* (> 75% of plasmalogens) and plasmalogen-deficient strains (less than 5% of plasmalogens) were compared [78]. A plasmalogen-deficient strain was found to increase the content of unsaturated and cyclopropane FAs, as well as the ratio of PS to PE from 0.59 in the wild type to 0.70 in a plasmalogen-deficient strain.

These properties are especially important for bacteria, many of which live in extreme conditions. In addition, plasmalogens can have a number of other functions. While much is already known about the biophysical properties of plasmalogens in mammalian (human) cells [94], this is not true of anaerobic bacteria.

8. Plasmalogens and protection against oxidative stress

The claim that plasmalogens protect membranes from oxidative stress by acting as sacrificial antioxidants in vivo is relatively speculative; experimental results argue either in favor or against. Singlet oxygen has been shown to interact more rapidly in vitro with ether lipids than with other lipids [100,101].

Regarding signaling and diseases caused by ether lipids, we refer to a number of reviews. Paul et al. [102] describes the therapeutic use of plasmalogens, their biosynthesis in the peroxisome of mammalian cells and their role in signal transduction in both prokaryotes and eukaryotes. Plasmalogens induce changes in the permeability of membranes, allowing Ca^{2+} to flow through plasma channels. Consequently, it allows the translocation of Ca^{2+}-dependent enzymes and other secondary messengers for further cell signal transduction [103]. The review also describes the possibility of using them in the treatment of cancer. Ether lipid signaling in both prokaryotes and eukaryotes is described by Rangholia et al. [104]. More detailed signaling in bacteria is described below. Ether lipid signaling in bacteria was investigated in Gram-negative soil

organisms, myxobacteria (*Myxococcus xanthus* and *Stigmatella aurantiaca*). These bacteria produce fruiting bodies rich in ether lipids, which help in their subsequent differentiation into spores. Unusual plasmalogens such as 1-A-13-methyl-1-*Z*-tetradecenyl-2-(13-methyltetradecanoyl) glycerophosphatidylethanolamine are metabolized to the appropriate A-15:0/i15:0/i15:0-TAG derivative (1-A-(13-methyltetradecyl))-2,3-di-(13-methyltetradecanoyl) glycerol)) if insufficient nutrients are available. [105,106]. This TAG and other triacylglycerols were no longer synthesized after 24 h in the absence of nutrients, which correlated with a dramatically reduced development of fruiting bodies and spores. This result supports the hypothesis that the relevant TAGs are signaling molecules and not energy storage depots [107]. Therefore, the appropriate TAGs are thought to be the basic signaling molecules needed to perform the complex life cycles of myxobacteria.

9. Conclusion

We see the future of plasmalogen research mainly in the combination of two basic techniques. Firstly, genome sequencing, where the amino acid sequences of the respective enzymes responsible for the biosynthesis of plasmalogens can be further examined. To date, the genomes of more than 10,000 bacteria have been sequenced (as of March 22, 2021). This method was also used by Jackson et al. [31], who list more than 1900 bacteria in which plasmalogens could be identified based on the presence of the *pls* operon, which may signify plasmalogen production. The plasmalogen pathway in anaerobic bacteria was observed early in life's evolution under anaerobic conditions and it seems that the biosynthetic pathway for plasmalogen is oxygen-sensitive [5,27]. This hypothesis was confirmed by robust plasmalogen production under anaerobic conditions in a recombinant strain of *E. coli* harboring the pPlsCP plasmid, after induction with IPTG. Production of plasmalogen by the recombinant strain, however, was not detected under aerobic conditions after IPTG induction [31].

Secondly, the LC-MS method is ideally suited for two-dimensional separation. Several articles published in recent years have taken advantage of two-dimensional separation, where in the first dimension, lipid classes are separated by HILIC, and using high-efficiency columns, plasmalogen phospholipids can be directly separated from diacyl-phospholipids. A reverse phase column (most often C18) already provides separation into individual molecular species that can be identified, including regioisomers, i.e. P-16:0/18:1-PE versus P-18:1/16:0-PE. The use of enzymatic cleavage with phospholipase C and the conversion of phospholipids to diradyl-glycerols (alkyl-acyl, alkenyl-acyl and diacyl glycerols) allows the further use of special columns, e.g. with a chiral stationary phase. Modern chromatographic and mass spectrometric techniques will definitely contribute to the elucidation of plasmalogen structures, especially in organisms where their presence was not expected until recently, e.g. in aerobic bacteria. For example, in the genus *Lactobacillus*, which is a genus of Gram-positive, facultative anaerobic or microaerophilic bacteria from the phylum *Firmicutes*, an enzyme 2-hydroxyacyl-CoA dehydratase (NCBI Reference Sequence: WP_179394538.1) has been described. This enzyme is responsible for the biosynthesis of plasmalogens (see text and Fig. 2 above).

One of the first articles describing the effect of Covid-19 on plasmalogen content was published. Coronaviruses have lipid envelopes needed for their activity. The fact that coronavirus infection induces the formation of cubic membranes in host cells has not been taken into account in the development of antiviral therapies. Plasmalogens tend to form a non-lamellar phase, facilitating membrane fusion, and modulating the activity of membrane-bound proteins such as enzymes and receptors. Plasmalogen deficiency in the lungs is associated with cardiometabolic disorders including obesity and type 2 diabetes in humans. It was also found that the susceptibility of these patients to SARS-CoV-2, i.e. the formation of severe acute respiratory syndrome, increases the severity of the disease and associated treatment difficulties. A correlation was found between plasmalogen deficiency in the pulmonary surfactant of COVID-19 patients and the composition of the lipid membrane. Plasmalogens can be recommended as a potential anti-viral therapeutic component in severely infected individuals [108].

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