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ORIGINAL ARTICLE

Hydrophilic Interaction Liquid Chromatography: ESI–MS/MS of Plasmalogen Phospholipids from *Pectinatus* Bacterium

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Abstract Liquid chromatography–electrospray tandem mass spectrometry (LC/ESI–MS/MS) was used to analyze phospholipids from three species of the anaerobic beer-spoilage bacterial genus *Pectinatus*. Analysis of total lipids by HILIC (Hydrophilic Interaction Liquid Chromatography) column succeeded in separating diacyl- and plasmalogen phospholipids. Plasmalogens were then analyzed by means of the ESI–MS/MS and more than 220 molecular species of four classes of plasmalogens (PlsCho (choline plasmalogen), PlsEtn (ethanolamine plasmalogen), PlsGro (glycerol plasmalogen), and PlsSer (serine plasmalogen)) were identified. Major molecular species were c-p19:0/15:0 PlsEtn and PlsSer, which accounted for more than 4% of the total lipids.

Keywords *Pectinatus* · Plasmalogens · Liquid chromatography–electrospray tandem mass spectrometry · HILIC column

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Abbreviations

CID	Collision-induced dissociation
DMSZ	Deutsche Sammlung von Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
ECL	Equivalent chain length
FA	Fatty acid(s)
FAME	Fatty acid methyl ester(s)
GC-MS	Gas chromatography–mass spectrometry
HILIC	Hydrophilic Interaction Liquid Chromatography
LC/ESI–MS/MS	Liquid chromatography–electrospray ionization tandem mass spectrometry
PtdOH	Phosphatidic acid
PtdIns	Phosphatidylinositol
PlsCho	Choline plasmalogen
PlsEtn	Ethanolamine plasmalogen
PlsGro	Glycerol plasmalogen
PlsSer	Serine plasmalogen
RIBM	Research Institute of Brewing and Malting
RSD	Relative standard deviation
TLC	Thin layer chromatography

Introduction

Microbial contamination that can occur during the brewing process can negatively affect the process of beer production and the quality of the final product. The contaminants can include, besides the so-called wild yeasts and molds, a

number of bacterial genera [1–3]. Improvements in beer handling and bottling technology have resulted in a significant reduction in the oxygen content during the process and in packaged beer, which has permitted the growth of anaerobic beer-spoilage bacteria such as *Pectinatus*, *Megasphaera*, *Selenomonas* and *Zymophilus* [1–5].

These anaerobic beer-spoilage bacteria belong to the class *Clostridia* and the family Veillonellacea; despite their phylogenetic affiliation to Gram-positive bacteria the cells stain Gram-negative or Gram-variable [6, 7]. The bacteria *Pectinatus* and *Zymophilus* have been isolated only from breweries, and their natural environment is still unknown [5, 6, 8].

The anaerobic beer-spoilage bacterium *Pectinatus* has become a serious problem in the brewing industry because it can cause strong turbidity and sediment in beer and produces unpleasant volatile compounds, mostly acids, e.g. acetic, propionic, succinic, butyric, valeric and caproic acid and also acetoin and hydrogen sulfide [1–3]. As a result, bacterial spoilage activity can completely damage the beer taste and quality and thus cause serious financial problems to the brewery.

Detection of these spoilage bacteria in breweries is still based on traditional incubation on special diagnostic/selective media [2, 3], which is very time-consuming. For example, development of turbidity in beer forcing tests takes 4–6 weeks [5, 6]. Using the SMMP-medium (a selective medium for *Megasphaera* and *Pectinatus*) the incubation takes up to 14 days [5]. Further alternatives are fatty acids or *O*-alk-1′-enyl chains analysis [3], epifluorescence microscopy, immunofluorescence [6] or modern genetic methods such as PCR [5], fluorescence in situ hybridization [6] or oligonucleotide microarrays [9].

Plasmalogen is any derivative of *sn*-glycero-3-phosphoric acid that contains an *O*-alk-1′-enyl residue (sometimes named vinyl ether moiety or enol ether moiety, see Fig. 1) attached to the glycerol moiety at *sn*-1 position and a polar head made of a nitrogenous base, a glycerol, inositol, etc. moiety.

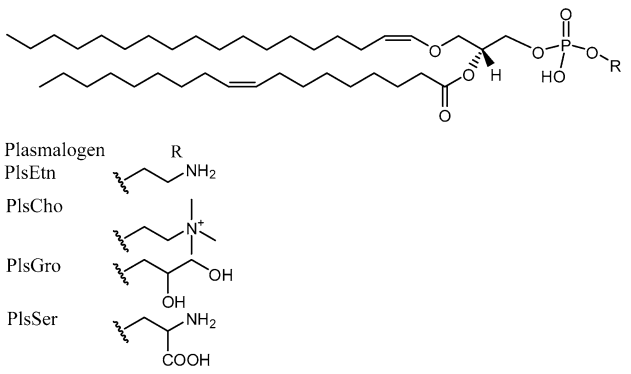


Fig. 1 The structures of plasmalogens. The explanation is in the abbreviations, the structures of the major ions is in Tables 2, 3, 4 and 5

Plasmalogens are among the most specific lipids since their occurrence is limited to strictly anaerobic bacteria [10] and they are not present in aerobic or facultatively anaerobic bacteria. They have been exceptionally found also in fungi [11] while their presence in higher plants is a matter of contention [12, 13]. By contrast, they are common constituents of animal lipids from invertebrates to humans [14]. They are found mostly as PlsCho (choline plasmalogen), PlsEtn (ethanolamine plasmalogen), PlsGro (glycerol plasmalogen), and PlsSer (serine plasmalogen), etc. (Fig. 1).

Analysis of plasmalogens makes use of their vinyl ether bond at the *sn*-1 position, which is liable to acid-catalyzed hydrolysis. The presence of mere traces of an acid causes hydrolysis resulting in a lysophospholipid and a fatty aldehyde. 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 alkyl-acyl, alkenyl-acyl and diacyl-glycerols by HPLC or, after derivatization of the free hydroxyl group, by GC [15]. 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 [15]. The authors [16–18] have always used an HPLC column with a diol bonded stationary phase and a highly complex organic phase consisting of up to six solvents [17] and, in addition, elution with a 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 HILIC (Hydrophilic Interaction Liquid Chromatography) column, although it has more often been used for separating water-soluble analytes [19]. Separation of phospholipids on a HILIC column is described in only a few papers, with relatively controversial results concerning especially the elution of individual phospholipid classes. Thus Schwalbe-Herrmann has described an elution order of PtdGro, PtdEtn and PtdCho [20], whereas Kamleh reported on the order PtdCh, PtdSer and PtdGro [21] and Scherer obtained the sequence PtdGro and PtdOH [22]. To our knowledge, our paper is the first to describe gradient HILIC separation of at least five phospholipid classes including plasmalogens.

Identification of intact plasmalogens has already been described by, e.g., Hsu et al. [23–26] who reported on ESI–MS or ESI–MS/MS identification as lithiated adducts, e.g.

$[M + Li]^+$ molecular ions and their cleavage to give characteristic fragments that allow identification of individual molecular species. The CID spectra of $[M + H]^+$ and $[M + Na]^+$ ions of PtdCho and PtdEtn are rather uninformative regarding the nature and position of the fatty acyl substituents in lipids [27]. In the presence of lithium salts, PtdCho and PtdEtn form $[M + Li]^+$ ions which, upon CID, produce product ion spectra that contain diagnostic fragment ions for both the polar head group and the fatty acyl groups.

This report is part of our investigation of analysis bacterial lipids within the framework of a comprehensive program on the analysis and biosynthesis of lipids [28, 29].

In this article, we present for the first time separation of intact plasmalogen phospholipids from diacyl phospholipids of anaerobic bacteria, and identification and quantitation of individual molecular species of four plasmalogens, i.e. PtdEtn, PtdGro, PtdSer, and PtdCho by LC-ESI/MS^2 from the three strains of bacterium *Pectinatus*.

Experimental

Bacterial Strains

Pectinatus frisingensis DSM 20465 and *P. cerevisiiphilus* DSM 20467 were purchased from the DSMZ Collection (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). *P. frisingensis* RIBM 2-86 was obtained from the RIBM Collection (Research Institute of Brewing and Malting, Prague, Czech Republic). All strains originated from brewery environment.

Cultivation and Extraction of Bacterium Strains

Pectinatus strains were grown in a modified MRS broth (medium according to de Man, Rogosa, and Sharpe, Merck, Darmstadt, Germany) supplemented with cysteine hydrochloride (0.5 g/L) and sodium thioglycollate (0.5 g/L), pH 5.6–5.7, for 48 h at 28 °C. Cultivation was performed under aerobic conditions (300 mL of modified MRS broth in 500-mL Erlenmeyer flasks, the surface of the medium was covered with sterile mineral oil to ensure anaerobiosis). No anaerobic atmosphere is needed if the medium is freshly prepared, is not stirred, the transfer of culture by pipette is quick and the inoculum is placed in the bottom of the test tube. After cultivation, the biomass was transferred to new tubes, chilled on ice and harvested immediately by centrifugation (10 min/6,000 rpm/0 °C). The tubes with pellets were placed on dry ice and stored frozen until freeze-dried. The yields of biomass and their lipid components are given in Table 1.

The extraction procedure was based on the method of Bligh and Dyer [30], except that 2-propanol was substituted for methanol, since 2-propanol does not serve as a substrate for phospholipases [31]. The alcohol–water mixture was cooled and one part chloroform was added and the lipids were extracted for 30 min. Insoluble material was sedimented by centrifugation and the supernatant was separated into two phases. The aqueous phase was aspirated off and the chloroform phase was washed three times with two parts 1 M KCl each. The resulting chloroform phase was evaporated to dryness under reduced pressure.

Table 1 Yields of biomass, total lipids and phospholipids of *Pectinatus frisingensis* DSM 20465, *P. cerevisiiphilus* DSM 20467, and *P. frisingensis* RIBM 2-86

	RIBM 2-86	DSM 20465	DSM 20467
Biomass in mg	306.0	407.0	469.0
Total lipids in mg	12.3	13.7	14.1
Total lipids in %	4.0	3.4	3.0
Phospholipids in mg	5.9	7.1	7.5
Phospholipids in % of total lipids	48.0	52.0	23.0

Data were obtained by weighing and by means of a Sep-Pak Cartridge with an aminopropylsilica-based polar bonded phase

First, total lipid extracts were applied to the Sep-Pak Cartridge Vac 35 cc (Waters; with 10 g of aminopropylsilica-based polar bonded phase), and from the cartridge were subsequently eluted nonacidic lipids with 40 mL of chloroform–methanol (7:3), and acidic lipids with 30 mL of chloroform–methanol-concentrated aqueous ammonia (70:30:2) containing 0.4% (w/v) ammonium acetate. The eluate was reduced in volume and subjected to semipreparative HILIC or LC–MS. No alkyl acyl species were present.

LC–MS Analysis and Semipreparative HILIC

The HPLC equipment consisted of a 1090 Win system, a PV5 ternary pump and an automatic injector (HP 1090 series, Hewlett Packard, USA). Gradient chromatographic separation was performed on a Merck ZIC®-HILIC HPLC column, with 3.5 µm 100 Å and 2.1 × 250 mm. The injection volume was 5 µL, and the column was maintained at 50 °C. The mobile phase consisted of (a) acetonitrile/water (8:2) with buffer (10 mM ammonium formate, 0.5 mM LiOH and 0.5 mM AcOLi) and (b) acetonitrile/water (5:5) with buffer (20 mM ammonium formate, 0.5 mM LiOH and 0.5 mM AcOLi). A gradient elution was performed with 100% A for 1 min, a step to 75% A until 5 min, a linear decrease to 50% A until 25 min, 50% A up to 10 min, and re-equilibration to 100% A for less than 5 min. The flow rate was set to 180 µL/min. The efficiency of the column was approximately 25,000 plates/250 mm.

The detector was an Applied Biosystems Sciex API 4000 mass spectrometer (Applied Biosystems Sciex, Ontario, Canada) using electrospray mass spectra. The ionization mode was positive, the nebulizing gas (N_2) pressure was 345 kPa and the drying gas (N_2) flow and temperature were 9 L/min and 300 °C, respectively. The electrospray needle was at ground potential, whereas the capillary tension was held at 3,900 V. The cone voltage was kept at 240 V. The mass resolution was 0.1 Da and the peak width was set to 6 s. For an analysis, total ion currents (full scan) were acquired from 200 to 1,100 Da.

CID ions mass spectra were acquired by colliding the Q1 selected precursor ions with Ar gas as a collision target gas and applying a collision energy of 50 eV in Q2. The scanning range of Q3 was *m*/*z* 150–1,100 with a step size of *m*/*z* 0.3 and a dwell time of 1 ms. A peak threshold of 0.3% intensity was applied to the mass spectra. The instrument was interfaced to a computer running Applied Biosystems Analyst version 1.4.1 software.

The liquid chromatograph was the semipreparative Gradient LC System G-1 (Shimadzu, Kyoto, Japan) with two LC-6A pumps (0.5 mL/min), an SCL-6A system controller, an SPD ultraviolet detector, an SIL-1A sample injector and a C-R3A data processor, with semipreparative normal phase HPLC ZIC®-HILIC 250 × 10 mm, 5 μm, 200 Å column. The gradient was as described above, with a flow rate of 4.5 mL/min, and was monitored by a variable wavelength detector at 210 nm (HP 1040 M Diode Array Detector).

Isolation of Aldehydes and Fatty Acids from Plasmalogens

In order to hydrolyze plasmalogens, the eluted compounds from semipreparative HILIC (∼ 10–100 μg) were treated with 90% acetic acid at 37 °C for 18 h and dried under reduced pressure. The products were dissolved in a small volume of chloroform and subjected to thin-layer chromatography on silica gel G, eluted by chloroform–methanol–7 M ammonia 60:35:5 (v/v/v) mixture and spots were located by spraying with water. The aldehydes, derived from the alk-l-enyl groups, and lysophospholipids were extracted from the plates with chloroform–methanol 1:2 (v/v). The fatty aldehydes were stored in hexane at −20 °C until analyzed. The lysophospholipid was heated at 75 °C in the presence of 10% NaOH and 50% methanol (1 mL) for 3 h. The hydrolysate was cooled, extracted twice with petroleum ether, and then adjusted to pH 3 with concentrated HCl. The acidified solution was extracted three times with diethyl ether (1 mL) and the combined extracts were then washed with water, and the sample was concentrated under nitrogen. The fatty acids were then methylated with diazomethane [32].

GC–MS

Gas chromatography–mass spectrometry of FAME and free aldehydes was done on a GC–MS system consisting of a Varian 450-GC, a Varian 240-MS ion trap detector with electron impact ionization, and a CombiPal autosampler (CTC, USA). The sample was injected onto a 25 m × 0.25 mm × 0.1 μm Ultra-1 capillary column (Supelco, Czech Republic) under a temperature program: 5 min at 50 °C, increasing at 10 °C/min to 280 °C and 15 min at 280 °C. Helium was the carrier gas at a flow of 0.52 °C/min. All spectra were scanned within the range *m*/*z* 50–500.

Results

Cells of three strains of genus *Pectinatus* were cultivated as described in Experimental and were harvested in log phase when the optical density of the culture was maximal. The time of cultivation was 48 h. The yield of lyophilized cells is shown in Table 1, which also gives the yield of lipids obtained by the Bligh–Dyer [30] extraction of lyophilized cells. Part of total lipids was fractionated by means of cartridges with aminopropyl silica-based polar bonded phase, which were rinsed in a chloroform–methanol mixture and phospholipids were further eluted by a mixture of chloroform–methanol–concentrated aqueous ammonia.

The HILIC employing a column with sulfobetaine group (3-(trimethylammonio)propane-1-sulfonate) with a zwitterionic character phase is suitable for separating phospholipids as well as their plasmalogen forms thanks to the use of an appropriate mobile phase, i.e. a buffer containing ammonium formate. This prevents acid hydrolysis of the enol bonds. Baseline resolution was achieved between all identified lipid classes, with partial resolution between alkenylacyl and diacyl phospholipids (Fig. 2) and Fig. 1 in Supplemental, describing the separation of five standards (16:0–18:1 PtdCho, 16:0–18:1 PtdEtn, 16:0–18:1 PtdSer, 16:0–18:1 PtdOH, and 16:0–18:1 PtdGro). The identities of alkenylacyl- and diacyl-phospholipid peaks were confirmed after HILIC separation followed by ESI–MS/MS, see below.

Baseline was stable and retention times of all identified classes of phospholipids were well reproducible, with standard deviations ± 0.05 min for the commercially obtained standards 1-*O*-1′-(*Z*)-octadecenyl-2-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphoethanolamine (p18:0/9*Z*-18:1-PtdEtn) and ± 0.07 min for 1-*O*-1′-(*Z*)-octadecenyl-2-(9*Z*-octadecenoyl)-*sn*-glycero-3-phosphocholine (p18:0/9*Z*-18:1-PtdCho).

Under these conditions, the main phospholipid classes were separated well. PtdGro was eluted first, followed by

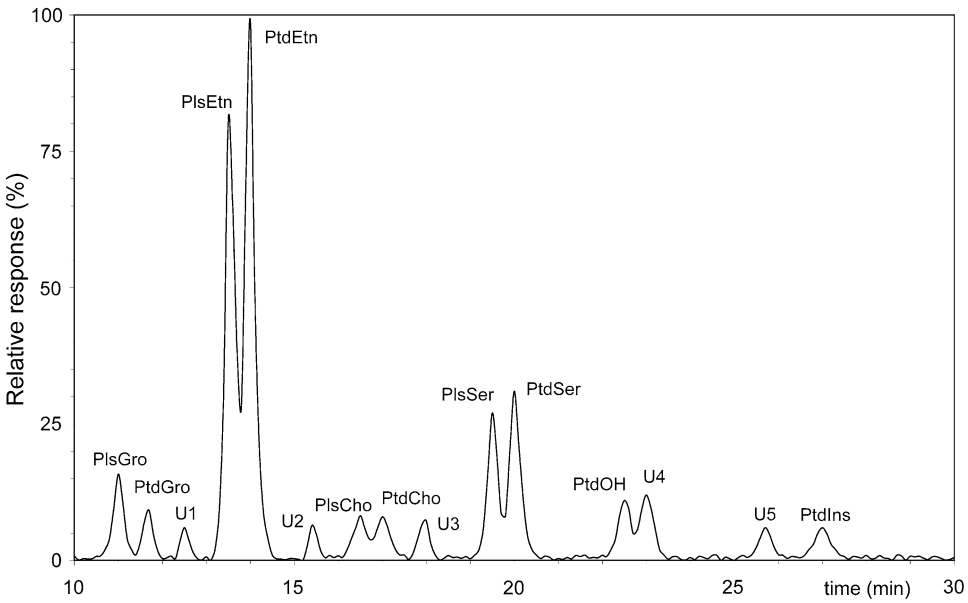


Fig. 2 HILIC/APCI-MS chromatogram of the phospholipids from *Pectinatus frisingensis* DSM 20465, analyzed by a linear gradient semipreparative normal phase HPLC ZIC®-HILIC 250 × 10 mm, 5 µm, 200-Å column, with a flow rate of 4.5 mL/min, and monitored by a variable wavelength detector at 210 nm

PtdEtn, PtdCho, PtdSer, PtdOH, and PtdIns (Fig. 2). Because different molecular species within a class have the same polar head, their retention times in HILIC are very similar. The differences in retention times for compounds within one class are less than that between two different classes. The different molecular species can be determined by MS/MS.

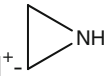
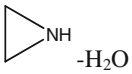
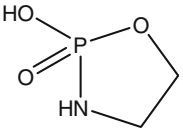
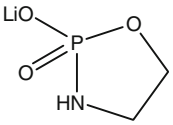
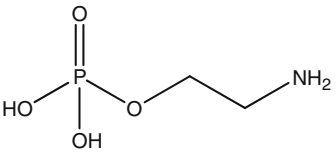
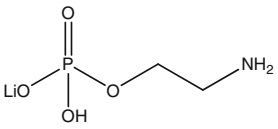
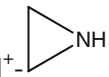
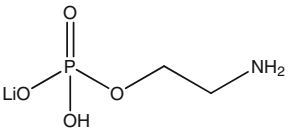
As mentioned in the Introduction, mass spectrometry fragmentation of diacylphospholipids has already been described but the situation with plasmalogen phospholipids is different. ESI tandem mass spectra of phospholipids as monolithiated $[M + Li]^+$ adduct molecular ions, arising at about 1 mM lithium salt concentrations in the mobile phase, show rich fragmentation, i.e. loss of some different neutral moieties, chiefly substituents at *sn*-3 or *sn*-2 position, exceptionally *sn*-1 (1-*O*-alk-1′-enyl) of plasmalogen. The corresponding ions are given in Tables 2, 3, 4 and 5. Apart from these ions that occur regularly in all four plasmalogens, some plasmalogens give rise to specific ions that can serve for direct identification of the given plasmalogen. Fast and exact identification was based on the separation by HILIC column and identification of phospholipids as monolithiated adducts, which allows for a ready identification of plasmalogens even in very low concentrations of about ~0.7 ng/µl.

The most abundant phospholipid was PtdEtn (29.7% total lipids) including its plasmalogen form (PlsEtn; 24.2%). Structural characterization of PlsEtn is complicated by numerous homologues that consist of various isomers (Fig. 3). Identification of individual molecular species was based on tandem MS. For example, the MS/MS spectrum of ion at *m/z* 678 of lithiated molecular species c-p17:0/15:1 PlsEtn showed the most abundant ion at *m/z* 635 ($[M + Li\text{-}43]^+$), i.e. ion with the structure shown in Fig. 4 and Table 2. Other ions present in the spectrum are formed by cleavage of neutral molecules, see e.g. ions at *m/z* 555 $[M + Li\text{-}123]^+$, *m/z* 549 $[M + Li\text{-}129]^+$, *m/z* 532 $[M + Li\text{-}141]^+$ and *m/z* 531 $[M + Li\text{-}147]^+$, arising from neutral losses; for structures see Table 2.

Two ions with moderate abundance at *m/z* 383, representing combined losses of aziridine and the *sn-1* alk-1′-enyl as an alcohol, i.e. $C_{15}H_{29}CH = CHOH$ and ion at *m/z* 291, representing the combined losses of lithium 2-aminoethyl hydrogenphosphate (for the structure see Table 2) and the *sn-2* fatty acid, i.e. pentadecenoic acid, were identified. The nature of the fatty acid at *sn*-2 was further verified by the presence of the acylium ion at *m/z* 240. Based on the above ions it is thus possible to determine in this plasmalogen the bond of the alkenyl ether at *sn-1* as well as the fatty acid at *sn-2*. The mass spectrum displays, below *m/z* 200, a dominant ion at *m/z* 148, which, though it does not provide information about the pPE structure, is nevertheless characteristic for PlsEtn and naturally also PtdEtn. This ion is not shown in the spectrum.

Identification of molecular species with the same molecular mass, i.e. isobaric species, was more complicated but not unfeasible, see Fig. 5. This can be best illustrated on two cases. First the mixture of two molecular species, i.e. c-p17:0/c-19:0 (5.5% of total PlsEtn) and c-p19:0/c-17:0 (4.8% of total PlsEtn), exhibits ions $[M + Li\text{-neutral molecules}]^+$, i.e. ions at *m/z* 691, 673, 611, 605, 593, and 587, which only confirm the structure of both molecular species and do not permit an even semi-quantitative estimate of their abundances. This estimate was only possible based on the cluster of cluster of four ions, i.e. two pairs (losses of aziridine with the *sn-1*

Table 2 Some important ions observed in the product ion spectrum of the $[M + Li]^+$ of molecular species c-p17:0/15:1-PlsEtn at *m/z* 678

PlsEtn	*m/z* for c-p17:0/15:1-PlsEtn
$[M+Li]^+$- 	678 − 43 = 635
$[M+Li]^+$-  -H_2O	678 − 43 − 18 = 617
*$[M+Li]^+$- 	678 − 123 = 555
*$[M+Li]^+$- 	678 − 129 = 549
$[M+Li]^+$- 	678 − 141 = 537
$[M+Li]^+$- 	678 − 147 = 531
$[M+Li]^+$-  -$R_1CH{=}CHOH$	678 − 43 − 252 = 383
$[M+Li]^+$-  -R_2COOH	678 − 147 − 240 = 291
R_2CO^+	223

Though Hsu and Turk [24] describe a linear structure of neutral fragments, i.e. $(HO)P(O)(OCH_2CH_2NH_2)$, a cyclic structure gives a better fit (see the neutral loss products in the paper by Zemski Berry and Murphy [50])

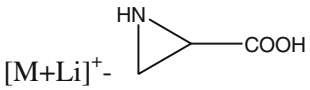
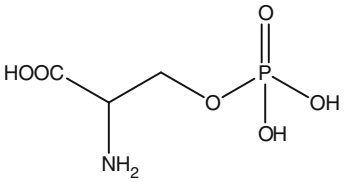
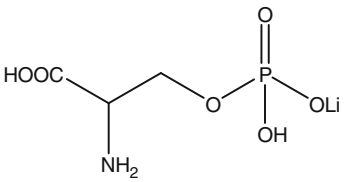
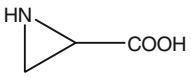
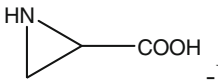
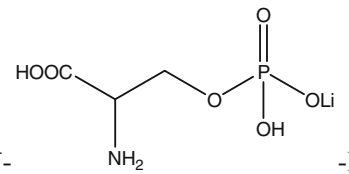
Data were obtained by RP-HPLC/ESI-tandem MS/MS operated in positive ionization mode

alk-1′-enyl and combined losses of lithium 2-aminoethyl hydrogen phosphate with the *sn-2* fatty acid. The abundance of ions at *m/z* 439 and 409, or those at *m/z* 319 and 291, enabled us to determine the semiquantitative abundances of individual molecular species.

The same holds for four molecular species, i.e. a most complex mixture of positional isomers containing these four molecular species, i.e. p15:0/c-17:0, p17:0/15:1, c-p17:0/15:0, and c-p19:0/13:0. Based on losses of aziridine with the *sn-1* alk-1′-enyl and combined losses of lithium 2-aminoethyl hydrogen phosphate with the *sn-2* fatty acid we could again determine the percent abundances. Further molecular species of next plasmalogens were identified similarly.

Like with the PlsEtn, also the tandem MS/MS spectrum of p17:0/15:0-PlsSer having $[M + Li]^+$ ion at *m/z* 726 contains prominent ions at *m/z* 541 and at *m/z* 535 arising from loss of phosphoserine as free acid and as a lithium salt, respectively (see Table 3). ESI spectrum of PlsGro is shown in Fig. 2 in Supplemental. The ion at *m/z* 639 (726-aziridine carboxylic acid) having medium abundance, and ions at *m/z* 397 (639-R_2COOH) and at *m/z* 415 (639-$R_2'CH = CO$) resulting from loss of the 15:0-fatty acid moiety and the corresponding loss of fatty acyl ketene, respectively, provide information needed for assignment of the *sn*-2 position of the fatty acyl substituents on the glycerol backbone. The ion at *m/z* 628 corresponding to loss of the phosphoric acid originated by rearrangement in which the anionic site of the serine head group makes a nucleophilic attack on C-3 of the glycerol backbone, followed by expulsion of the phosphoric acid. The mass spectrum also features an ion at *m/z* 484 arising from $[M + Li]^+$ loss of the fatty acid group. The presence of ion at *m/z* 281 confirms the position of the alkenyl ether at *sn*-1. The structure of fatty acid bonded to glycerol backbone was again confirmed by the presence of ion $[R_2CO]^+$

Table 3 Some important ions observed in the product ion spectrum of the $[M + Li]^+$ of molecular species p17:0/15:0 PlsSer at *m/z* 726

PlsSer	*m/z* for p17:0/15:0 PlsSer
 $[M+Li]^+$-	726 − 87 = 639
$[M + Li]^+$-H_3PO_4	726 − 98 = 628
 $[M+Li]^+$-	726 − 185 = 541
 $[M+Li]^+$-	726 − 191 = 535
$[M + Li]^+$-R_2COOH	726 − 242 = 484
 $[M+Li]^+$- -R´$_2$CH=C=O	726 − 87 − 224 = 415
 $[M+Li]^+$- -R_2COOH	726 − 87 − 242 = 397
 $[M+Li]^+$- -R´$_1$CH=CHOH	726 − 191 − 214 = 321
R_2CO^+	225

Data were obtained by RP-HPLC/ESI-tandem MS/MS operated in positive ionization mode

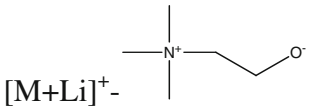
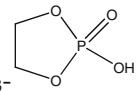
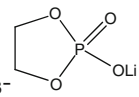
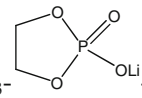
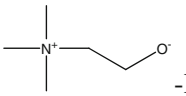
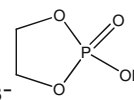
at *m/z* 225. Further, the spectrum displayed an ion at *m/z* 192 corresponding to a lithiated phosphoserine ion via cleavage of the C_3-OP bond. This ion (not shown in our spectrum) is characteristic only for PtdSer and therefore also for PlsSer.

The qualitative and quantitative abundance of molecular species having the same summary formula and thus the same molecular weight, i.e. c-p19:0/18:1 and p18:1/c-19:0, was analyzed as described with PlsEtn. Hence we can determine the abundance of two ions at *m/z* 423 and at *m/z* 409, i.e. ion with structure [M-aziridine carboxylic acid-fatty acid]$^+$ and two appropriate ions at *m/z* 335 and at *m/z* 321 with structure [M-phosphoserine as lithium salt-$R_1'CH = CHOH]^+$. Semiquantitative abundance of three or four molecular species with the same summary formula, e.g. c-p19:0/15:0, p18:1/16:1, c-p17:0/17:0, and p17:0/c-17:0, was estimated as described above with PlsEtn.

The c-p17:0/15:1 PlsCho molecular species having $[M + Li]^+$ ion at *m/z* 720 yields a prominent product ion at *m/z* 661 caused by loss of $N(CH_3)_3$ (Table 4). For the ESI spectrum of PlsCho from *Pectinatus frisingensis* see Fig. 3 in the Supplemental Material. This *m/z* 661 $[M + Li-59]^+$ ion yields ions of *m/z* 537 $[M + Li-183]^+$ and *m/z* 531 $[M + Li-189]^+$ resulting from losses of ethylene phosphate and lithium ethylene phosphate, respectively. Identification of the fatty acid substituent at *sn*-2 is based on the occurrence of the *m/z* 472 ion $[M + Li-R_2COOLi]^+$ that is produced by loss of lithium pentadecanoate from *m/z* 720, and a prominent ion of *m/z* 291 $[531-C_{16}H_{31}COOH]^+$ caused by loss of pentadecenoic acid from *m/z* 531. The pentadecanoate moiety is clearly identified by the presence of the acylium ion at *m/z* 223 $[C_{16}H_{31}CO]^+$. Weak ions at *m/z* 468 and 409, which identify the 1-*O*-heptadecenyl moiety, arise by loss of the 1-alkenyl moiety as an alcohol ($C_{13}H_{27}CH = CHOH$) from *m/z* 720 and 661, respectively, and further dissociation of the latter ion yields a weak ion at *m/z* 395.

The last plasmalogen was identified as PlsGro; its ESI spectrum is shown in Fig. 4 in Supplemental. The tandem MS/MS spectrum of an ion $[M + Li]^+$ at *m/z* 793 indicates

Table 4 Some important ions observed in the product ion spectrum of the $[M + Li]^+$ of molecular species c-p17:0/15:1-PlsCho at *m/z* 720

PlsCho	*m/z* for c-p17:0/15:1-PlsCho
$[M + Li]^+$-NMe_3	720 − 59 = 661
	720 − 103 = 617
$[M+Li]^+$-	
	720 – 59 − 124 = 537
$[M+Li]^+$-NMe_3-	
	720 – 59 − 130 = 531
$[M+Li]^+$-NMe_3-	
	720 – 59 − 130 − 18 = 513
$[M+Li]^+$-NMe_3- -H_2O	
$[M + Li]^+$- $R'_1CH = CHOH$	720 − 252 = 468
$[M + Li]^+$-R_2COOLi	720 − 248 = 472
$[M + Li]^+$-NMe_3- $R'_1CH = CHOH$	720 − 59 − 252 = 409
	720 − 103 − 222 = 395
$[M+Li]^+$- -$R'_2CH=C=O$	
	720 − 59 − 130 − 240 = 291
$[M+Li]^+$-NMe_3- -R_2COOH	
R_2CO^+	223

Data were obtained by RP-HPLC/ESI-tandem MS/MS operated in positive ionization mode

the structure of p19:0/c-19:0 PlsGro. Loss of dihydroxycyclopropane from this $[M + Li]^+$ ion gives rise to a prominent ion at *m/z* 719, see Table 4. Loss of fatty acid from this ion yields in turn an ion at *m/z* 423, which is important for the diagnostics of the structure of fatty acid at *sn*-2. Also the ion at *m/z* 497, arising by a mere loss of fatty acid from the $[M + Li]^+$, determines the structure of fatty acid at *sn*-2. Like PtdSer, also PtdGro molecule undergoes a rearrangement with a concomitant expulsion of phosphoric acid, giving rise to the ion $[M + Li\text{-}H_3PO_4]^+$ at *m/z* 695. As mentioned in the Introduction, many ions are common for all phospholipids; in the case of PtdGro these are the ions arising by splitting of the polar part of molecule form *sn*-3 of glycerol backbone, i.e. ions at *m/z* 621 and at *m/z* 615. These ions arise by the splitting of glycerophosphoric acid (systematic name is 2,3-dihydroxypropyl dihydrogen phosphate) or its lithium salt $[M + Li\text{-}172]^+$ and $[M + Li\text{-}178]^+$, respectively. For structure of both ions see Table 5.

The remaining two phospholipids, i.e. PtdOH and PtdIns, in which no plasmalogens have been found, were identified based on literature data, see below. After identifying all four plasmalogens we performed quantitation of fatty acids and phospholipids in the three strains of genus *Pectinatus*.

Phospholipids were further separated by semipreparative HILIC, see Experimental. The major phospholipids were PtdGro, PtdEtn, PtdCho, and PtdSer, including their plasmalogens; PtdIns was present in minor amounts. Five peaks were not identified, see Fig. 3.

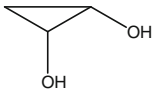
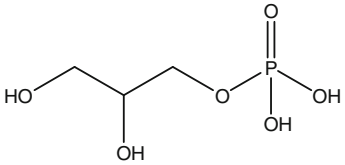
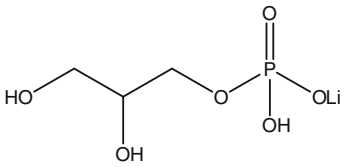
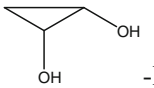
The content of fatty acids determined by GC–MS in the three species of *Pectinatus* is given in Table 6. The fatty acids included saturated ones from C11 to C18, as well as monoenoic ones and also two acids having a cyclopropane ring in the midchain position. The quantitative proportion of FA in different phospholipids of one species, as well as in individual strains did not substantially differ.

A similar situation was found in the case of aldehydes arising by mild acidic hydrolysis; an interesting feature was the minor proportion of octadecenal in all three PlsEtn.

Discussion

The comparison of our results with the literature data is difficult or nearly impossible. As was found already in the

Table 5 Some important ions observed in the product ion spectrum of the $[M + Li]^+$ of molecular species p19:0/c-19:0-PlsGro at *m/z* 793

PlsGro	*m/z* for p19:0/c-19:0-PlsGro
 $[M+Li]^+$-	793 − 74 = 719
$[M + Li]^+$-H_3PO_4	793 − 98 = 695
 $[M+Li]^+$-	793 − 172 = 621
 $[M+Li]^+$-	793 − 178 = 615
$[M + Li]^+$-R_2COOH	793 − 296 = 497
 $[M+Li]^+$- -R_2COOH	793 − 74 − 296 = 423
R_2CO^+	279

Data were obtained by RP-HPLC/ESI-tandem MS/MS operated in positive ionization mode

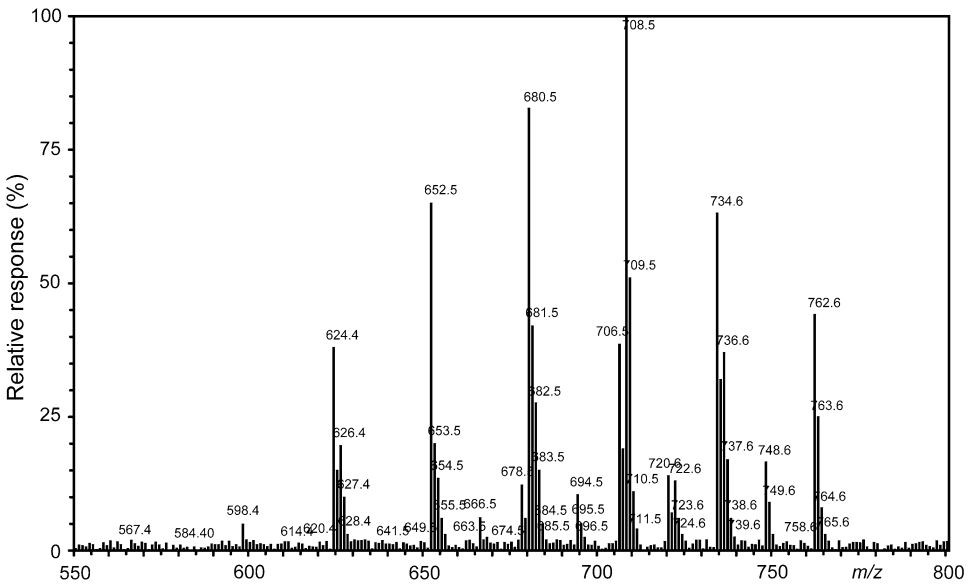


Fig. 3 ESI spectrum of PlsEtn from *Pectinatus frisingensis* DSM 20465. MS was run by using the electrospray mode and set to scan from 200 to 1,100 kDa. Only the most relevant part of the mass spectrum is shown (550–800 kDa). Adduct ions in the form of $[M + Li]^+$ are seen throughout the spectrum

1960s, fatty acids with a cyclopropane ring are very susceptible to acidic conditions that cause cleavage of the ring. This was documented in 1965 by Nesbitt and Lennarz [33] who therefore saponified cells of the genus *Proteus* by 10% NaOH and methylated free FA by diazomethane. This approach, which was used also by other authors [34], avoided the cleavage of the cyclopropane ring. As noted in 1989 by Christie in his book, "Cyclopropane fatty acids of bacterial origin are more susceptible to chemical attack, e.g. cyclopropane ring is disrupted by acidic conditions, and lipid samples containing such acids are best transesterified with basic reagents; the free fatty acids can be methylated safely with diazomethane" [35]. The dramatic drop in the content of cyclopropane FA under acidic conditions when preparing FAME was recently amply documented by Basconcillo and McCarry [36]. The table published in their paper shows that basic hydrolysis and acid esterification of FA from the bacterium *Sinorhizobium meliloti* brings about a drop of cis-11,12-C19:0_{cyclo} from 31 to 2.5% total FA, i.e. by more than one order of magnitude.

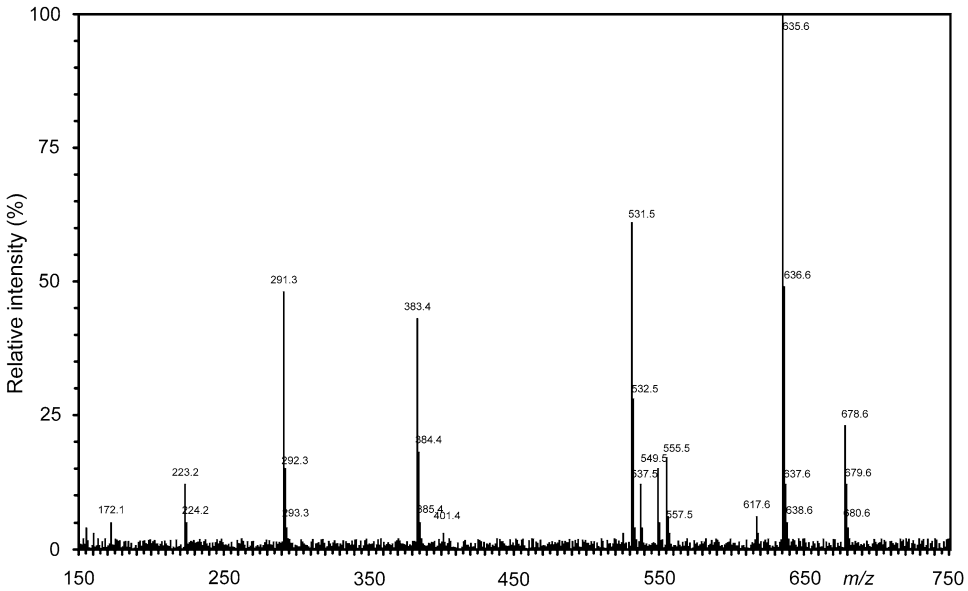


Fig. 4 The MS/MS spectrum of lithiated molecular species c-p17:0/15:1 PlsEtn obtained in ESI. The ion at *m/z* 678.6 was selected for fragmentation. This ion was further fragmented with CID resulting in the presented spectrum. Fragment identities and their structures are explained in Table 2

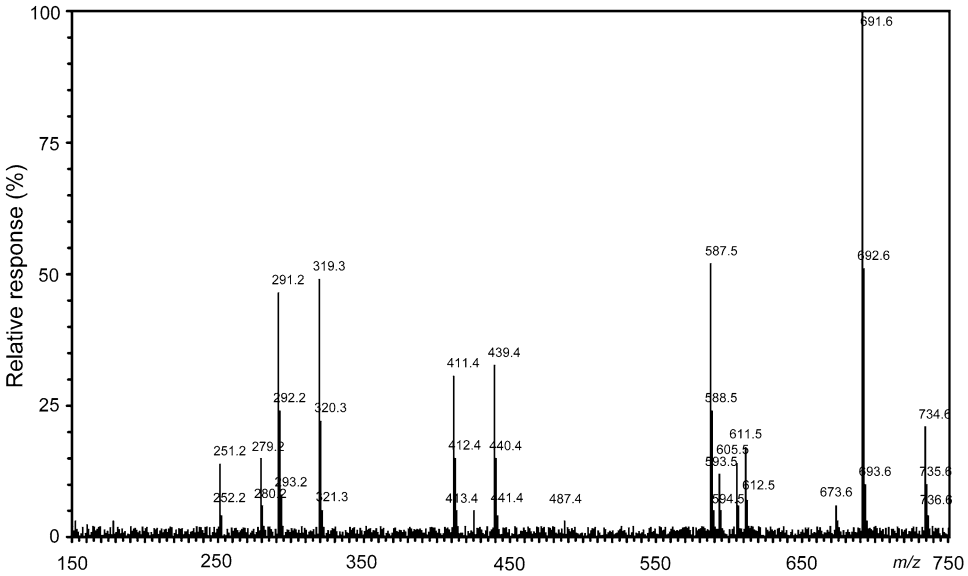


Fig. 5 Tandem MS/MS of the mixture of two molecular species—c-p17:0/c-19:0 and c-p19:0/c-17:0 PlsEtn. Quantification of isobaric species of plasmalogens was based on six ions, i.e. ions at *m/z* 251 and 279 $[RCO]^+$, *m/z* 291.3 and 319.3 $[M + Li\text{-}147\text{-}R'2COOH]^+$, and *m/z* 411.4 and 439.4 $[M + Li\text{-}43\text{-}R'1CH = CHOH]^+$

Accordingly, Moore et al. [37], who used basic hydrolysis and acid-catalyzed esterification, found no cyclopropane FA in *Pectinatus*. Gonzalez et al. [38], who erroneously identified the species *P. portalensis*, also failed to find any cyclo-FA, not even in the previously described *P. frisingensis* or *P. cerevisiiphilus*. Moreover, their data showed completely baffling differences between the contents of FA in individual species. Johnston and Goldfine [39], who analyzed the related genus *Megasphaera*, realized that the chromatographic peak of monoenoic FAME could contain cyclopropane FAME and found that, following catalytic reduction that disrupts the cyclopropane ring, major c-17:0 acyl chain peak decreased from 60.5 to 2.9% of total FA. This means that it was in fact a mixture of two FA, i.e. cyclo-17:0 and 17:1 in a ratio of about 20:1, not a pure 17:1.

Unusual ions arising from 9-methoxy acids and found in the MS spectrum by Helander and Haikara [3] led to the conclusion that these acids were artificially created during methanolysis from cyclopropane acids. The substantial differences between FAME prepared by methanolysis and those prepared by saponification can thus be explained by the degradation of cyclopropanoic acids.

Even when using reaction conditions preventing degradation of cyclopropane FA, the experimenter is faced with two other problems. As has been known for several decades [35], the ECL values of C18 cyclopropane FAME and corresponding C18 monoenoic methyl esters are approximately equal, see, e.g., Fig. 2 in the paper by Helander and Haikara [3]. Moreover, cyclopropanoid FAME are not readily distinguished by MS from monoenoic FAME with a

Table 6 Fatty acid content of the three strains of *Pectinatus frisingensis* DSM 20465, *P. cerevisiiphilus* DSM 20467, and *P. frisingensis* RIBM 2-86

	P. frisingensis RIBM 2-86				*P. cerevisiiphilus* DSM 20467				*P. frisingensis* DSM 20465			
	PlsEtn	PlsSer	PlsGro	PlsCho	PlsEtn	PlsSer	PlsGro	PlsCho	PlsEtn	PlsSer	PlsGro	PlsCho
11:0	18.1	16.4	16.5	22.6	17.4	16.8	17.1	21.1	14.7	12.2	14.2	13.8
12:0	0.8	0.3	0.4	0.8	1.2	0.0	0.0	1.5	0.8	0.3	0.8	0.4
13:0	6.2	6.8	5.3	8.6	6.3	6.7	5.1	8.5	6.5	6.9	8.2	5.6
14:0	1.7	0.6	1.0	1.6	2.1	0.2	0.5	2.2	1.8	0.6	1.5	1.1
15:0	27.1	26.2	17.6	15.1	25.9	27.2	18.3	14.3	26.3	31.4	27.5	28.6
16:0	1.8	0.3	2.2	1.5	2.2	0.0	1.8	2.2	1.9	0.3	1.4	2.3
17:0	5.0	3.9	4.7	2.0	5.2	3.7	4.5	2.6	5.3	4.0	1.9	5.0
18:0	1.2	2.0	1.6	1.7	1.6	1.6	1.1	2.3	1.3	2.0	1.6	1.7
15:1	5.9	3.0	5.8	7.4	6.0	2.7	5.7	7.4	6.2	3.0	7.1	6.1
16:1	0.8	1.1	1.3	4.0	1.2	0.7	0.8	4.4	0.8	1.1	3.8	1.4
c-17:0	10.6	16.0	14.9	16.6	10.4	16.5	15.4	15.7	10.5	12.2	11.4	11.6
18:1	4.8	8.7	6.2	5.7	5.0	8.8	6.1	5.9	5.0	8.8	5.4	6.6
c-19:0	16.0	14.7	22.5	12.4	15.5	15.1	23.6	11.9	18.9	17.2	15.2	15.8
p15:0	4.6	0.0	5.3	2.3	3.2	2.1	7.2	2.4	4.9	0.0	2.4	5.5
p17:0	16.0	11.2	18.7	12.4	14.8	18.6	6.8	10.6	27.8	11.4	12.8	22.7
c-p17:0	34.2	33.2	20.9	23.8	25.9	25.8	29.7	30.1	27.6	25.4	24.6	21.6
p18:1	0.0	20.2	3.6	19.4	4.9	10.2	12.6	6.3	0.2	20.5	20.0	3.7
c-p19:0	45.2	35.4	51.5	42.1	51.2	43.3	43.7	50.6	39.5	42.7	40.2	46.5

0.0 = less than 0.1%

Data were obtained by GC–MS of fatty acid methyl esters, the structure of unsaturated fatty acids was determined by the mass spectra of picolinyl esters

similar total number of carbon atoms, apparently because on ionisation the cyclopropane ring opens up to form some degradation products [40, 41]. Much better results are obtained with picolinyl ester derivatives (see Harvey [42] and also our two mass spectra given in Figs. 5 and 6 of Supplemental).

Most authors analyzing the *Pectinatus* FA failed to employ optimum conditions and the reliability of their data is therefore questionable. The same holds for monoenoic and cyclopropanoic aldehydes.

Quantification of individual classes of phospholipids including plasmalogens is based on the total ion current of commercially available standards. The calibration curves of four standards, i.e. PtdCho, PlsCho, PtdEt and PlsEtn (9*Z*-18:1/9*Z*-18:1-PtdCho, p18:0/9*Z*-18:1-PlsCho, 9*Z*-18:1/9*Z*-18:1-PtdEtn, and p18:0/9*Z*-18:1-PlsEtn) showed that these molecular species have a linear response in the range of nearly four orders of magnitude, i.e. from 1 μmol/mL to 10 mmol/mL (i.e. ~0.7 μg/mL to ~7 mg/mL). The worst values of % RSD (relative standard deviation) were obtained for the lowest and the highest chromatographic peaks. This could be due to the signal being too weak (a low signal to noise ratio) or to a charge space phenomenon caused by the accumulation of too many ions in the mass spectrometer (in the case of the highest chromatographic peak, i.e. at a high concentration of the compound). The samples were therefore appropriately diluted to an optimal concentration (100 μmol/mL) to avoid the loss of signal from the lowest chromatographic peak and limit the charge space phenomenon for the highest chromatographic peak. This compromise maximizes reproducibility. Unfortunately, PlsGro and PlsSer are not commercially available but both these plasmalogens can be assumed to have a very similar mechanism of ionization (see above) allowing thus also the same quantification by means of the total ion current. Comparison of relative concentrations of individual FAME calculated from GC/MS and from HPLC/MS-APCI of TAGs is given in Table 1 in Supplemental.

The quantification of single ion molecular species was much more complicated. Based on the measurements of commercially available standards PlsCho and PlsEtn and the corresponding homologs PtdCho and PtdEtn (i.e. 9*Z*-18:1/9*Z*-18:1-PtdCho and 9*Z*-18:1/9*Z*-18:1-PtdEtn, respectively) we found that the abundant ions at *m/z* 425 $[M + Li\text{-}43\text{-}R'_1CH = CHOH]^+$ and at *m/z* 305 $[M + Li\text{-}147\text{-}R'_2COOH]^+$ in PlsEtn and ions at *m/z* $[M + Li\text{-}43\text{-}R_1COOH]^+$ and $[M + Li\text{-}43\text{-}R_2COOH]^+$ in PtdEtn, and also ions $[M + Li\text{-}59\text{-}R_1COOH]^+$ and $[M + Li\text{-}59\text{-}R_2COOH]^+$ in PtdCho and ions at *m/z* 465 ($[M + Li\text{-}59\text{-}R'_1CH = CHOH]^+$) and at *m/z* 395 ($[M + Li\text{-}103\text{-}R'_2$

$CH = C{=}O]^+$) in PlsCho show a linear response in an interval of more than three orders of magnitude, i.e. from 5 to 10 mmol/mL. Individual molecular species of plasmalogens were therefore quantified based on the above ion. Because of the lack of commercial standards of PlsGro and PlsSer we again used the assumption of a very similar mechanism of ionization. More exact quantification would require either the use of two-dimensional HPLC (normal and reversed phase HPLC) or acquisition (synthesis) of appropriate standards, i.e. different molecular species of PlsSer and PlsGro.

As already mentioned in the Introduction and in the paragraph describing the occurrence of fatty acids in *Pectinatus* and related bacteria, the determination of both qualitative and quantitative proportions of FA is highly questionable. To our knowledge, no paper devoted to identification and quantitation of molecular species of plasmalogens in *Pectinatus* has as yet been published. The results of our analyses of these molecular species are shown in Table 7. Although the molecular species of individual classes of phospholipids have been described in bacteria such as *Escherichia coli* [43] or *Corynebacterium* [44], these bacteria are taxonomically relatively distant from *Pectinatus*. The taxonomically nearest bacterial genus that has been described in the literature is *Clostridium*, which also belongs to anaerobes and is taxonomically classed to the same order (Clostridiales), although to a different family (Clostridiaceae) [45].

Both our literature survey and a recent review [15] point to the scarcity of data on the analysis of molecular species of phospholipids, especially of plasmalogens in bacteria. Only a single study by Hsu and Turk [43] reported on the analysis of cardiolipin from *E. coli* using modern techniques, identified cardiolipin and described the quantitative analysis of isobaric molecular species.

Only six molecular species of *O*-alanylphosphatidylglycerol and six molecular species of phosphatidylethanolamine were identified in the genus *Clostridium*, which

Table 7 The content of molecular species of plasmalogens in the three strains of *Pectinatus frisingensis* DSM 20465, *P. cerevisiiphilus* DSM 20467, and *P. frisingensis* RIBM 2-86

Molecular species		*P. frisingensis* RIBM 2-86				*P. cerevisiiphilus* DSM 20467				*P. frisingensis* DSM 20465			
		PlsEtn	PlsSer	PlsGro	PlsCho	PlsEtn	PlsSer	PlsGro	PlsCho	PlsEtn	PlsSer	PlsGro	PlsCho
p15:0	/11:0	0.8	0.0	0.0	0.5	0.0	0.7	0.3	0.8	0.6	0.4	1.2	0.5
p17:0	/11:0	2.9	1.8	1.8	2.8	1.4	4.1	1.8	3.1	2.6	3.1	1.2	2.2
c-p17:0	/11:0	6.2	5.4	5.4	5.4	3.1	4.1	3.5	3.0	4.5	4.3	5.1	6.4
p18:1	/11:0	0.0	3.3	3.3	4.4	2.5	0.0	2.8	0.5	0.9	1.7	2.2	1.3
c-p19:0	/11:0	8.2	5.8	5.8	9.5	5.2	5.8	5.7	6.4	8.9	7.3	7.5	10.7
p15:0	/12:0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
p17:0	/12:0	0.1	0.0	0.0	0.1	0.0	0.2	0.1	0.1	0.2	0.0	0.0	0.2
c-p17:0	/12:0	0.3	0.1	0.1	0.2	0.1	0.2	0.2	0.1	0.3	0.0	0.0	0.5
p18:1	/12:0	0.0	0.1	0.1	0.2	0.1	0.0	0.2	0.0	0.1	0.0	0.0	0.1
c-p19:0	/12:0	0.4	0.1	0.1	0.3	0.1	0.3	0.3	0.2	0.6	0.0	0.0	0.8
p15:0	/12:0	0.3	0.0	0.0	0.2	0.0	0.3	0.2	0.3	0.2	0.1	0.4	0.2
p17:0	/13:0	1.0	0.8	0.8	1.1	0.8	1.8	1.0	1.3	0.9	1.2	0.3	0.9
c-p17:0	/13:0	2.1	2.3	2.3	2.0	1.8	1.8	2.0	1.2	1.6	1.7	1.5	2.6
p18:1	/13:0	0.0	1.4	1.4	1.7	1.4	0.0	1.6	0.2	0.3	0.7	0.6	0.5
c-p19:0	/13:0	2.8	2.4	2.4	3.6	2.9	2.6	3.3	2.6	3.2	2.9	2.2	4.3
p15:0	/14:0	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.0	0.0	0.1
p17:0	/14:0	0.3	0.1	0.1	0.2	0.1	0.5	0.2	0.2	0.3	0.0	0.0	0.2
c-p17:0	/14:0	0.6	0.2	0.2	0.4	0.2	0.5	0.4	0.2	0.5	0.1	0.1	0.7
p18:1	/14:0	0.0	0.1	0.1	0.3	0.1	0.0	0.3	0.0	0.1	0.0	0.1	0.1
c-p19:0	/14:0	0.8	0.2	0.2	0.7	0.3	0.7	0.6	0.5	1.1	0.1	0.2	1.1
p15:0	/15:0	1.2	0.0	0.0	0.3	0.0	1.3	0.7	1.6	0.8	0.6	1.3	0.3
p17:0	/15:0	4.3	2.9	2.9	1.9	3.6	7.3	3.5	6.5	3.8	5.1	1.2	1.5
c-p17:0	/15:0	9.3	8.7	8.7	3.6	8.0	7.3	6.8	6.2	6.7	7.0	5.4	4.3
p18:1	/15:0	0.0	5.3	5.3	2.9	6.4	0.1	5.5	1.1	1.3	2.8	2.3	0.9
c-p19:0	/15:0	12.2	9.3	9.3	6.4	13.4	10.4	11.1	13.3	13.3	11.8	8.4	7.2
p15:0	/16:0	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.0	0.1	0.1

Table 7 continued

Molecular species		*P. frisingensis* RIBM 2-86				*P. cerevisiiphilus* DSM 20467				*P. frisingensis* DSM 20465			
		PlsEtn	PlsSer	PlsGro	PlsCho	PlsEtn	PlsSer	PlsGro	PlsCho	PlsEtn	PlsSer	PlsGro	PlsCho
p17:0	/16:0	0.3	0.0	0.0	0.2	0.0	0.5	0.2	0.5	0.3	0.0	0.1	0.2
c-p17:0	/16:0	0.6	0.1	0.1	0.4	0.1	0.5	0.3	0.5	0.6	0.0	0.5	0.7
p18:1	/16:0	0.0	0.1	0.1	0.3	0.1	0.0	0.3	0.1	0.1	0.0	0.2	0.1
c-p19:0	/16:0	0.8	0.1	0.1	0.6	0.1	0.8	0.6	1.1	1.1	0.0	0.8	1.1
p15:0	/17:0	0.2	0.0	0.0	0.0	0.0	0.3	0.0	0.3	0.2	0.1	0.3	0.1
p17:0	/17:0	0.8	0.4	0.4	0.2	0.5	1.5	0.2	1.1	0.8	0.7	0.3	0.3
c-p17:0	/17:0	1.7	1.3	1.3	0.5	1.0	1.5	0.5	1.1	1.3	1.0	1.3	0.8
p18:1	/17:0	0.0	0.8	0.8	0.4	0.8	0.0	0.4	0.2	0.3	0.4	0.6	0.2
c-p19:0	/17:0	2.3	1.4	1.4	0.8	1.7	2.1	0.8	2.3	2.7	1.6	2.0	1.3
p15:0	/18:0	0.1	0.0	0.0	0.0	0.0	0.1	0.0	0.1	0.1	0.0	0.1	0.1
p17:0	/18:0	0.2	0.2	0.2	0.2	0.2	0.4	0.2	0.4	0.2	0.3	0.1	0.2
c-p17:0	/18:0	0.4	0.7	0.7	0.4	0.5	0.4	0.4	0.4	0.4	0.4	0.3	0.7
p18:1	/18:0	0.0	0.4	0.4	0.3	0.4	0.0	0.3	0.1	0.1	0.2	0.1	0.1
c-p19:0	/18:0	0.5	0.7	0.7	0.7	0.9	0.5	0.6	0.8	0.8	0.7	0.5	1.2
p15:0	/15:1	0.3	0.0	0.0	0.2	0.0	0.3	0.2	0.3	0.2	0.1	0.4	0.2
p17:0	/15:1	0.9	0.3	0.3	0.9	0.3	1.7	0.9	1.4	0.9	0.5	0.4	0.8
c-p17:0	/15:1	2.0	1.0	1.0	1.8	0.8	1.7	1.7	1.3	1.6	0.7	1.7	2.2
p18:1	/15:1	0.0	0.6	0.6	1.4	0.6	0.0	1.4	0.2	0.3	0.3	0.7	0.5
c-p19:0	/15:1	2.7	1.1	1.1	3.1	1.3	2.4	2.9	2.8	3.1	1.2	2.5	3.7
p15:0	/16:1	0.0	0.0	0.0	0.1	0.0	0.0	0.1	0.1	0.0	0.0	0.1	0.1
p17:0	/16:1	0.1	0.1	0.1	0.5	0.1	0.2	0.5	0.3	0.2	0.1	0.1	0.5
c-p17:0	/16:1	0.3	0.4	0.4	1.0	0.3	0.2	0.9	0.3	0.3	0.2	0.2	1.3
p18:1	/16:1	0.0	0.2	0.2	0.8	0.2	0.0	0.8	0.1	0.1	0.1	0.1	0.3
c-p19:0	/16:1	0.4	0.4	0.4	1.7	0.5	0.3	1.5	0.7	0.6	0.3	0.3	2.2
p15:0	/c-17:0	0.5	0.0	0.0	0.4	0.0	0.5	0.3	0.6	0.3	0.3	1.1	0.4
p17:0	/c-17:0	1.7	1.8	1.8	2.1	1.4	2.9	1.5	2.6	1.5	3.1	1.0	1.7
c-p17:0	/c-17:0	3.6	5.3	5.3	4.0	3.1	2.9	2.8	2.5	2.7	4.3	4.6	4.7
p18:1	/c-17:0	0.0	3.2	3.2	3.2	2.5	0.0	2.3	0.4	0.5	1.7	1.9	1.0
c-p19:0	/c-17:0	4.8	5.7	5.7	7.0	5.2	4.1	4.6	5.4	5.3	7.1	6.7	7.9
p15:0	/18:1	0.2	0.0	0.0	0.1	0.0	0.2	0.1	0.4	0.2	0.2	0.4	0.1
p17:0	/18:1	0.8	1.0	1.0	0.7	1.0	1.4	0.7	1.5	0.7	1.6	0.4	0.6
c-p17:0	/18:1	1.6	2.9	2.9	1.4	2.2	1.4	1.3	1.4	1.3	2.3	1.8	1.8
p18:1	/18:1	0.0	1.8	1.8	1.1	1.8	0.0	1.1	0.2	0.2	0.9	0.8	0.4
c-p19:0	/18:1	2.2	3.1	3.1	2.4	3.8	2.0	2.2	3.1	2.6	3.8	2.7	2.9
p15:0	/c-19:0	0.7	0.0	0.0	0.3	0.0	0.9	0.4	0.9	0.5	0.3	1.7	0.3
p17:0	/c-19:0	2.6	1.6	1.6	1.5	2.0	5.3	1.9	3.6	2.3	2.8	1.6	1.3
c-p17:0	/c-19:0	5.5	4.9	4.9	3.0	4.4	5.2	3.7	3.4	3.9	3.9	7.0	3.6
p18:1	/c-19:0	0.0	3.0	3.0	2.4	3.5	0.0	3.0	0.6	0.8	1.4	3.0	0.7
c-p19:0	/c-19:0	7.2	5.2	5.2	5.2	7.3	7.5	6.1	7.3	7.9	6.5	10.3	6.0

Data were obtained from ESI spectra

belongs to the same order as the genus *Pectinatus*, although the polar lipids of *C. perfringens* were analyzed by LC/MS with electrospray ionization. The assumed presence of plasmalogens was based only on a TLC plate treated with HCl vapors, lyso-phosphatidylethanolamine being derived by acid hydrolysis of the plasmalogens of PtdEtn [46].

A later work on *C. tetani* identified phospholipids such as PtdEtn, PtdGro, cardiolipin and EtnPGlcNAcdiradylglycerol. FA on *sn*-1/*sn*-2 were determined only in

seven out of 35 molecular species of PE and PG, no data on their quantitative proportions being given [47].

The high variability of bacteria can be illustrated in the paper [48] in which plasmalogens were identified in *C. tetani* ATCC 454 but not in strain ATCC 10779. The authors again identified plasmalogen forms of PtdEtn, PtdGro, cardiolipin, etc., but acyls at the *sn*-1 and *sn*-2 positions were determined in only 7 of them, again with no quantification.

An excellent paper on phospholipids and also plasmalogens of lower organisms was published in Parasitology [49]. Many hundreds of molecular species of phospholipids were identified in parasitic protist species *Trypanosoma brucei*, which included, e.g., 128 molecular species of PtdEtn, out of which 15 were plasmalogens.

Conclusion

The HILIC/ESI-MS^n method used here efficiently separated plasmalogens and all other phospholipids usually found in anaerobic bacteria by a single chromatographic run. We determined more than 200 lipid molecular species from four lipid classes with one injection on an HILIC column, which is nearly ideal for separating amphiphilic compounds such as phospholipids with excellent reproducibility, high sensitivity (from 1 μmol/mL) and dynamic range (4 orders of magnitude). This column was used for both analytical determination and semipreparative isolation of alkenyl-acyl- and diacyl-phospholipids. Irrespective of the class of phospholipids, plasmalogen is eluted first and this is followed by a nearly baseline-separated corresponding diacyl-phospholipid. The elution can be performed conveniently by an aqueous phase with a buffer compatible with ESI.

Simultaneous detection of intact plasmalogens together with other phospholipid classes (odd-chains fatty acids, aldehydes) offers 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 offers an optimum means 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 greatly aid in the differentiation of the plasmenyl- and diacyl-phospholipid subclasses, including PtdEtn, PtdCho, PtdGro and PtdSer. Thus, structures of plasmalogens with isobaric isomers in mixtures can be unambiguously unveiled.

We believe that the method will be valuable for studying phospholipid profiles in different biological samples.

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References

1. Jespersen L, Jakobsen M (1996) Specific spoilage organisms in breweries and laboratory media for their detection. Food Microbiol 33:139–155
2. Sakamoto K, Konings WN (2003) Beer spoilage bacteria and hop resistance. Int J Food Microbiol 89:105–124
3. Basařová G, Šavel J, Basař P, Lejsek T (2010) Brewing, theory and practice of beer production (Pivovarství, teorie a praxe výroby piva). Vydavatelství VŠCHT Praha, Prague, pp 310–348
4. Helander IM, Haikara A (1995) Cellular fatty acyl and alkenyl residues in *Megasphaera* and *Pectinatus* species: contrasting profiles and detection of beer spoilage. Microbiology 141:1131–1137
5. Satokari R, Juvonen R, Mallison K, von Wright A, Haikara A (1998) Detection of beer spoilage bacteria *Megasphaera* and *Pectinatus* by polymerase chain reaction and colorimetric microplate hybridization. Int J Food Microbiol 45:119–127
6. Juvonen R, Koivula T, Haikara A (2008) Group-specific PCR-RFLP and real-time PCR methods for detection and tentative discrimination of strictly anaerobic beer-spoilage bacteria of the class *Clostridia*. Int J Food Microbiol 125:162–169
7. Schleifer KH, Leuteritz M, Weiss N, Ludwig W, Kirchhof G, Sedel-Rüfer H (1990) Taxonomic study of anaerobic, gram-negative, rod-shaped bacteria from breweries: Emended description of *Pectinatus cerevisiiphilus* and description of *Pectinatus frisingensis* sp. nov., *Selenomonas lacticifex* sp. nov., *Zymophilus raffinosivorans* gen. nov., sp. nov., and *Zymophilus paucivorans* sp. nov. Int J Syst Bacteriol 40:19–27
8. Matoulková D (2008) Strictly anaerobic bacteria in beer and in breweries. Kvasny Prum 54:338–343
9. Weber DG, Sahm K, Polen T, Wendisch VF, Antranikian G (2008) Oligonucleotide microarrays for the detection and identification of viable beer spoilage bacteria. J Appl Microbiol 105:951–962
10. Takatsuka Y, Kamio Y (2004) Molecular dissection of the *Selenomonas ruminantium* cell envelope and lysine decarboxylase involved in the biosynthesis of a polyamine covalently linked to the cell wall peptidoglycan layer. Biosci Biotechnol Biochem 68:1–19
11. Horrocks LA, Sharma M (1982) Plasmalogens and O-alkyl glycerophospholipids. In: Hawthorne JN, Ansell GB (eds) Phospholipids. Elsevier, Amsterdam, pp 51–93
12. Felde R, Spiteller G (1994) Search for plasmalogens in plants. Chem Phys Lipids 71:109–113
13. Mangold HK (1972) The search for alkoxylipids in plants. In: Snyder F (ed) Ether lipids: chemistry and biology. Academic Press, New York, pp 399–405
14. Goldfine H (2010) The appearance, disappearance and reappearance of plasmalogens in evolution. Prog Lipid Res 49:493–498

15. Christie WW, Han X (2010) Lipid analysis, 4th edn. The Oily Press, Bridgwater
16. Uran S, Larsen A, Jacobsen PB, Skotland T (2001) Analysis of phospholipid species in human blood using normal-phase liquid chromatography coupled with electrospray ionization ion-trap tandem mass spectrometry. J Chromatogr B 758:265–275
17. Olsson NU, Harding AJ, Harper C, Salem N (1996) High-performance liquid chromatography method with light scattering detection for measurements of lipid class composition: analysis of brains from alcoholics. J Chromatogr B 681:213–218
18. Mawatari S, Okuma Y, Fujino T (2007) Separation of intact plasmalogens and all other phospholipids by a single run of high-performance liquid chromatography. Anal Biochem 370:54–59
19. Nguyen HP, Schug KA (2008) The advantages of ESI-MS detection in conjunction with HILIC mode separations: fundamentals and applications. J Sep Sci 31:1465–1480
20. Schwalbe-Herrmann M, Willmann J, Leibfritz D (2010) Separation of phospholipid classes by hydrophilic interaction chromatography detected by electrospray ionization mass spectrometry. J Chromatogr A 1217:5179–5183
21. Kamleh A, Barrett MP, Wildridge D, Burchmore RJS, Scheltema RA, Watson DG (2008) Metabolomic profiling using Orbitrap Fourier transform mass spectrometry with hydrophilic interaction chromatography: a method with wide applicability to analysis of biomolecules. Rapid Commun Mass Spectrom 22:912–1918
22. Scherer M, Schmitz G, Liebisch G (2010) Simultaneous quantification of cardiolipin, bis(monoacylglycero)phosphate and their precursors by hydrophilic interaction LC-MS/MS including correction of isotopic overlap. Anal Chem 82:8794–8799
23. Hsu FF, Turk J (2009) Electrospray ionization with low-energy collisionally activated dissociation tandem mass spectrometry of glycerophospholipids: mechanisms of fragmentation and structural characterization. J Chromatogr B 877:2673–2695
24. Hsu FF, Turk J (2000) Characterization of phosphatidylethanolamine as a lithiated adduct by triple quadrupole, tandem mass spectrometry with electrospray ionization. J Mass Spectrom 35:596–606
25. Hsu FF, Turk J (2005) Studies on phosphatidylserine by tandem quadrupole and multiple stage quadrupole ion-trap mass spectrometry with electrospray ionization: Structural characterization and the fragmentation processes. J Am Soc Mass Spectr 16:1510–1522
26. Hsu FF, Turk J, Thukkani AK, Messner MC, Wildsmith KR, Ford DA (2003) Characterization of alkylacyl, alk-1-enylacyl and lyso subclasses of glycerophosphocholine by tandem quadrupole mass spectrometry with electrospray ionization. J Mass Spectrom 38:752–763
27. Hsu FF, Turk J (2005) Electrospray ionization with low-energy collisionally activated dissociation tandem mass spectrometry of complex lipids: structural characterization and mechanisms of fragmentation. In: Byrdwell WC (ed) Modern methods for lipid analysis by liquid chromatography/mass spectrometry and related techniques. AOCS Press, Champaign, pp 61–178
28. Lisa M, Holcapek M, Rezanka T, Kabatova N (2007) High-performance liquid chromatography-atmospheric pressure chemical ionization mass spectrometry and gas chromatography-flame ionization detection characterization of delta 5-polyenoic fatty acids in triacylglycerols from conifer seed oils. J Chromatogr A 1146:67–77
29. Rezanka T, Mares P, Husek P, Podojil M (1986) Gas-chromatography mass-spectrometry and desorption chemical ionization mass-spectrometry of triacylglycerols from the green-alga *Chlorella kessleri*. J Chromatogr 355:265–271
30. Bligh EG, Dyer WJ (1959) A rapid method of total lipid extraction and purification. Can J Biochem Physiol 37:911–917
31. Kates M (1986) Techniques of lipidology: isolation, analysis and identification of lipids. In: Work TS, Work E (eds) Laboratory techniques in biochemistry and molecular biology, 2nd edn. Elsevier, Amsterdam, pp 220–223
32. Khuller GK, Goldfine H (1974) Phospholipids of *Clostridium butyricum*. V. Effects of growth temperature on fatty alk-1-enyl ether group, and phospholipid. J Lip Res 15:500–507
33. Nesbitt JA, Lennarz WJ (1965) Comparison of lipids and lipopolysaccharide from the bacillary and L forms of *Proteus* P18. J Bacteriol 89:1020–1025
34. Khuller GK, Goldfine H (1974) Phospholipids of *Clostridium butyricum*. V. Effects of growth temperature on fatty acid, 1-alkenyl ether group, and phospholipid composition. J Lipid Res 15:500–507
35. Christie WW (1989) Gas chromatography and lipids. A practical guide, Oily Press Ltd
36. Basconcillo LS, McCarry BE (2008) Comparison of three GC/MS methodologies for the analysis of fatty acids in *Sinorhizobium meliloti*: development of a micro-scale, one-vial method. J Chromatogr B 871:22–31
37. Moore LVH, Bourne DM, Moore WEC (1994) Comparative distribution and taxonomic value of cellular fatty acids in thirty-three genera of anaerobic Gram-negative bacilli. Int J Syst Bact 44:338–347
38. Gonzalez JM, Jurado V, Laiz L, Zimmermann J, Hermosin B, Saiz-Jimenez C (2004) *Pectinatus portalensis* nov sp., a relatively fast-growing, coccoidal, novel *Pectinatus* species isolated from a wastewater treatment plant. Antonie van Leeuwenhoek 86:241–248
39. Johnston NC, Goldfine H (1982) Effects of growth temperature on fatty acid and alk-1-enyl group compositions of *Veillonella parvula* and *Megasphaera elsdenii* phospholipids. J Bacteriol 149:567–575
40. Christie WW, Holman RT (1966) Mass spectrometry of lipids. I. Cyclopropane fatty acid esters. Lipids 1:176–182
41. Wood R, Reiser R (1965) Cyclopropane fatty acid metabolism; physical and chemical identification of propane ring metabolic products in the adipose tissue. J Am Oil Chem Soc 42:315–320
42. Harvey DJ (1984) Picolinyl derivatives for the characterization of cyclopropane fatty acids by mass spectrometry. Biomed Mass Spectrom 11:187–192
43. Hsu FF, Turk J (2006) Characterization of cardiolipin from *Escherichia coli* by electrospray ionization with multiple stage quadrupole ion-trap mass spectrometric analysis of [M–2H + Na]-ions. J Am Soc Mass Spectr 17:420–429
44. Mazzella N, Molinet J, Syakti AD, Barriol A, Dodi A, Bertrand JC, Doumenq P (2005) Effects of pure n-alkanes and crude oil on bacterial phospholipid classes and molecular species determined by electrospray ionization mass spectrometry. J Chromatogr B 822:40–53
45. de Vos P, Garrity G, Jones D, Krieg NR, Ludwig W, Rainey FA, Schleifer KH, Whitman WB (eds) (2009) Bergey's manual of systematic bacteriology. Vol 3: The Firmicutes, Springer
46. Johnston NC, Baker JK, Goldfine H (2004) Phospholipids of *Clostridium perfringens*: a reexamination. FEMS Microbiol Lett 233:65–68
47. Mazzella N, Molinet J, Syakti AD, Dodi A, Doumenq P, Artaud J, Bertrand JC (2004) Bacterial phospholipid molecular species analysis by ion-pair reversed-phase HPLC/ESI/MS. J Lipid Res 45:1355–1363
48. Johnston NC, Aygun-Sunar S, Guan Z, Ribeiro AA, Daldal F, Raetz CRH, Goldfine H (2010) A phosphoethanolamine-modified glycosyl diradylglycerol in the polar lipids of *Clostridium tetani*. J Lipid Res 51:1953–1961
49. Richmond GS, Gibellini F, Young SA, Major L, Denton H, Lilley A, Smith TK (2010) Lipidomic analysis of bloodstream and procyclic form *Trypanosoma brucei*. Parasitology 137:1357–1392

50. Zemski Berry KA, Murphy RC (2004) Electrospray ionization tandem mass spectrometry of glycerophosphoethanolamine plasmalogen phospholipids. J Am Soc Mass Spectr 15:1499–1508
51. Holčapek M, Lísa M, Jandera P, Kabátová N (2005) Quantitation of triacylglycerols in plant oils using HPLC with APCI-MS, evaporative light-scattering, and UV detection. J Sep Sci 28:1315–1333 (this paper is cited in Supplementary material 1)
52. Rezanka T (1990) Identification of very long polyenoic acids as picolinyl esters by Ag+ ion-exchange high-performance liquid-chromatography, reversed-phase high-performance liquid-chromatography and gas-chromatography mass-spectrometry. J Chromatogr 513:344–348 (this paper is cited in Supplementary material 1)