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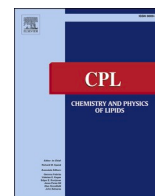
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A versatile method to separate complex lipid mixtures using 1-butanol as eluent in a reverse-phase UHPLC-ESI-MS system

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ABSTRACT

Simple, robust and versatile LC-MS based methods add to the rapid assessment of the lipidome of biological cells. Here we present a versatile RP-UHPLC-MS method using 1-butanol as the eluent, specifically designed to separate different highly hydrophobic lipids. This method is capable of separating different lipid classes of glycerophospholipid standards, in addition to phospholipids of the same class with a different acyl chain composition. The versatility of this method was demonstrated through analysis of lipid extracts of the bacterium *Escherichia coli* and the archaeon *Sulfolobus acidocaldarius*. In contrast to 2-propanol-based methods, the 1-butanol-based mobile phase is capable of eluting highly hydrophobic analytes such as cardiolipins, tetraether lipids and mycolic acids during the gradient instead of the isocratic purge phase, resulting in an enhanced separation of cardiolipins and extending the analytical range for RPLC.

1. Introduction

Cellular membranes fulfil the essential function of compartmentalization, separating cellular contents from the extracellular environment and enable the formation and maintenance of specialized reaction compartments. Membranes are predominantly formed by glycerophospholipids that due to their amphipathic characteristics can self-assemble into bilayer structures. Glycerophospholipids that constitute the membrane of Archaea are markedly different from those found in Bacteria and Eukarya. One of the defining features of archaeal glycerophospholipids is the presence of ether-bound isoprenoid-based lipid tails instead of ester-bound acyl lipid tails found in bacteria and eukaryotes. This feature makes archaeal lipids more hydrophobic compared to their fatty-acid based counterparts. Various archaea also synthesize more complex glycerophospholipids such as cardiolipins (CLs) or membrane spanning tetraether lipids that resemble two tail-to-tail dimerized diether lipids forming a macrocyclic structure (Corcelli et al., 2000; Corcelli, 2009; Schouten et al., 2007). These lipids in particular are strongly hydrophobic and therefore can complicate chromatographic analysis using traditional reversed-phase LC methods.

The study of archaeal lipid membranes and the mechanisms of biosynthesis conveys information about what ancient cell membranes might have looked like and provide the basis for the Tex86 historical temperature proxy that is extensively used to predict surface temperatures in the Cenozoic era (Schouten et al., 2002).

Due to high sample complexity, contemporary lipidomics often requires multi-dimensional analysis (Rezanka et al., 2014). This is typically facilitated by methods such as LC-MS, a powerful tool allowing for detailed sample analysis (Holčapek et al., 2012). Shotgun lipidomics by direct infusion MS has been documented as well and has the advantage of short analysis times facilitating high-throughput studies. Although, the lack of analyte separation can cause ion suppression, interfering with the identification of low abundance lipids (Yang and Han, 2011). Furthermore, the lack of analyte separation can interfere with absolute quantification and requires the use of a mass filter prior to MS/MS analysis for structural characterization (Jensen et al., 2015a; Schuhmann et al., 2012; Jensen et al., 2015b; Wang and Han, 2019). The low resolution of such mass filters (~0.4 Da) leaves analysis methods that have no analyte separation, and therefore rely on “MS-only” information, susceptible to interference of isobaric lipids. Thus these methods

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are generally less suitable for detailed structural characterization of lipids in highly complex samples containing isobaric or isomeric lipid species (Yang and Han, 2011; Rustam and Reid, 2018; Züllig et al., 2020).

Methods of lipid separation are usually based on normal-phase (NP) or reversed-phase (RP) LC (Wörmer et al., 2013). Other methods employ 2-dimensional separation for more comprehensive analysis. These methods combine various separation techniques and select these techniques on the basis of desired separation selectivity, compatibility and throughput (Lísa and Holčapek, 2013; Holčapek et al., 2009; Yang et al., 2020; Lísa and Holčapek, 2015). NPLC is often used to analyze environmental samples and generally uses relatively apolar mobile phase solvent mixtures consisting of 2-propanol (2-PrOH), hexane, chloroform and small amounts of water. This is used in combination with bare silica or diol-columns to separate lipids into different lipid classes, mostly based on polar head group hydrophilicity (Sollai et al., 2019; Sturt et al., 2004; Van Mooy et al., 2009; Scherer et al., 2010; Van Mooy et al., 2006). The separation of lipids into classes is particularly useful for biomarker studies, hence NPLC methods are more commonly used in environmental biology. An NPLC-based technique, hydrophilic interaction liquid chromatography (HILIC), is commonly employed for lipidomic analyses (Hájek et al., 2017; Lísa et al., 2017; Shen et al., 2020; Pham et al., 2019; Hines et al., 2017). HILIC uses a hydrophilic stationary phase, like NPLC; with solvents that are less hydrophobic, often employing an acetonitrile (MeCN)-water gradient, and thus are more comparable to RPLC mobile phase compositions. As with NPLC, HILIC methods tend to separate lipids according to their class. However, lipids often elute in a relatively narrow time window, resulting in a single chromatographic peak per lipid class in which lipid species can only be distinguished by mass. This property greatly benefits quantification of lipid species, as a single internal lipid standard per class is sufficient to correct for a difference in response factor for that class (Cifková et al., 2012; Lísa et al., 2017; Yang and Han, 2011).

In contrast to NPLC-based techniques, RPLC uses hydrophobic stationary phases, most commonly C18-modified silica combined with more polar solvent mixtures such as water, MeCN and 2-PrOH. This difference in solvents and column material shifts the selectivity of RPLC methods to separate lipids mostly based on the hydrophobic properties of the lipid tail. Separation based on polar headgroup characteristics still takes place, but to a much lower degree compared to NPLC-based techniques (Řezanka et al., 2014; Wörmer et al., 2013; Rampler et al., 2018; Cajka and Fiehn, 2016; Oursel et al., 2007; Damen et al., 2014; Breitkopf et al., 2017; Triebel et al., 2017).

While most RPLC methods are suitable for the separation of fatty acids, lyso-glycerophospholipids and diester glycerophospholipids; the separation of highly hydrophobic lipids, such as cardiolipins or archaeal tetraether lipids can be challenging for water/MeCN- and water/2-PrOH-based methods. These highly hydrophobic lipids are difficult to elute from C18 columns, which often results in co-elution, peak broadening and in more extreme cases even leading to an absence of signal. These issues limit analytical coverage of RPLC and can be particularly problematic for the analysis of complex biological samples. As these samples contain many different lipids, which can have very different concentrations to one another; co-elution can obscure low abundance lipids, or interfere with reliable quantification due to ion suppression effects. Another common problem in the study of lipids through LC-MS is the co-elution of isomeric lipids (Řezanka et al., 2014; Rustam and Reid, 2018; Oursel et al., 2007; Jeucken et al., 2019; Scherer et al., 2011). For instance, cis-trans isomeric lipids cannot be distinguished by common mass spectrometry techniques and thus isomer analysis largely relies on chromatographic separation techniques (Rustam and Reid, 2018; Bird et al., 2012; Gunstone et al., 1967). However, while not commonplace at this time, ion mobility spectrometry (IMS) techniques are gaining in popularity for lipidomic analyses. IMS offers an extra orthogonal source of information in addition to chromatographic separation and MS (Paglia and Astarita, 2017; Lintonen et al., 2014). This

technique is able to distinguish and separate isomeric lipids based on their conformational shape, despite often having identical retention times, providing more information and confidence for lipid identification and lipidomic characterization (Šala et al., 2016; Groessl et al., 2015; Bowman et al., 2017; Vasilopoulou et al., 2020).

Classic normal-phase or HILIC methods do not always have the desired resolution and selectivity that RPLC methods have to offer for a given set of analytes; for example, lipidomic analyses requiring in-depth structural characterization for which a large variety of acyl tails or isoprene chains of varying length or saturation needs to be detected and separated. In addition, most LC systems are configured to run under RPLC conditions and switching to an NPLC configuration can involve significant downtime. Despite these drawbacks, normal-phase chromatographic separation excels in the separation of similar ether-lipid cores and has the advantage of allowing stronger injection solvents (Schouten et al., 2007; Shimada et al., 2008; Shimada et al., 2002; Damsté et al., 2007; Hopmans et al., 2000). To increase the eluotropic range of RPLC with the aim to separate more hydrophobic lipids, we increased the eluotropic strength of the secondary mobile phase compared to established methods by using 1-butanol (1-BuOH) as a solvent. Here we present a versatile RP-UHPLC-ESI-MS method capable of separating complex biological lipid mixtures, including especially hydrophobic molecules such as the isoprene-based cardiolipin molecules and tetraether lipid species that are often found in archaeal lipid membranes.

2. Materials and methods

2.1. Growth conditions and cell harvesting

Escherichia coli DH5 α was cultured under aerobic conditions at 37 °C with 200 RPM shaking. A pre-heated 4 L Erlenmeyer flask containing 1 L of LB medium was inoculated to OD₆₀₀ 0.01 and incubated for 16 h (OD₆₀₀ 2.5). *Sulfolobus acidocaldarius* MW001 was cultured under aerobic conditions at 76 °C with 200 RPM shaking in Brock medium (Wagner et al., 2012; Brock et al., 1972). A pre-heated 1 L Erlenmeyer flask containing 500 mL of Brock medium pH 3 supplemented with 10 μ g/mL uracil was inoculated to OD₆₀₀ 0.03 and incubated to OD₆₀₀ 1.5 over 70 h. Cells were harvested by centrifugation at 7,500 \times g for 15 min at 4 °C and washed with 15 mL buffer (50 mM Tris-HCl pH 8, 100 mM KCl). Washed cells were pelleted (4,000 \times g, 15 min at 4 °C) and flash frozen in liquid N₂. Frozen cell pellets were placed in a pre-cooled freeze dryer set to -55 °C at 0.01 mbar pressure and left to dry for 3 days.

2.2. Total lipid extraction and sample preparation

Lipids were extracted from 10 mg freeze-dried cells from *E. coli* DH5 α or *S. acidocaldarius* MW001 pellets using an adapted acidic Bligh and Dyer method employing 0.1 M HCl instead of 2 M HCl or 5 % trichloroacetic acid as described elsewhere (Bligh and Dyer, 1959; Nishihara and Koga, 1987). The crude chloroform fraction was dried and the lipid film was three times re-extracted using 400 μ L chloroform. The resulting fraction was dried, three times re-extracted with 400 μ L chloroform - methanol (1:2), dried again and finally three times re-extracted with 400 μ L methanol. The resulting lipid film was dissolved to 5 mg/mL in methanol and filtered through a 0.2 μ m PTFE syringe filter prior to analysis.

All pure lipid stocks and the *E. coli* cardiolipin extract (Avanti polar lipids, cat: 841199) were dissolved in chloroform. To prepare these samples for analysis, chloroform containing a measured amount of lipids (or a mix thereof) was evaporated under a N₂-stream at 37 °C to form a lipid film. This film was then re-dissolved in methanol (to 0.25 mg/mL for pure lipids or 0.1 mg/mL for lipid standard mixtures) for UHPLC-ESI-MS analysis.

2.3. Reverse-phase UHPLC method development

For the separation of lipids, Waters C18 charged surface hybrid (CSH) and bridged ethylene hybrid (BEH) (150 × 2.1 mm internal diameter) columns with a 1.7 μm particle size were selected (Wörmer et al., 2013; Damen et al., 2014). Typically, RPLC methods for lipid analysis employ mixtures of MeCN, methanol and ultrapure water (MQ) as eluent A. 2-PrOH typically forms the basis of eluent B. To increase the eluotropic strength of eluent B, in-house distilled 1-BuOH (Sigma Aldrich, cat#: 901351, supplementary methods) was selected as solvent for its stronger interaction with lipids compared to 2-PrOH. Ammonium formate (5 mM) was employed as buffering agent to promote negative ion formation; as lipids such as phosphatidic acid (PA) and phosphatidylethanolamine (PE) tend to ionize more easily in negative mode. The addition of aqueous ammonia to the mobile phase of a similar method has been reported to improve peak shape (Wörmer et al., 2013). However, aqueous ammonia was not included to avoid retention time increases, lipid-class specific ionization efficiency alterations and reduced column lifetime. A mixture of MeCN and MQ was selected for the composition of eluent A. To provide sufficient retention for less hydrophobic analytes, such as lyso-phospholipids, up to 40 % of MQ was added. This was sufficient to allow for separation of dodecyl maltoside (DDM) and lyso-phosphatidic acid (LPA) and lyso-phosphatidyl glycerol (LPG) without causing miscibility issues when combined with eluent B. In turn, eluent B was modified to contain 0.5 % MQ to allow for buffer solubilization and 10 % MeCN to prevent miscibility problems with eluent A. Ultimately, the analytes in a standard mixture (DDM; 18:1/18:1 PG, PC, PE, PA; 18:1/18:1/18:1/18:1 CL and AG) responded virtually identical to both the CSH and BEH columns. The intended purpose of this method was to separate many different lipids in complex samples, such as total lipid extracts. As these samples contain a large variety of analytes, the C18 CSH column was selected to further develop the method to reduce the potential risk of peak shape issues for compounds not included in our standard samples. At 60 °C the peak width of the standard compounds eluting from the C18 CSH column was found to be between 1.5 and 2 times smaller compared to the peak width at 40 °C. In favor of column lifetime, we decided to use 55 °C for a better balance between manufacturer recommended operating parameters and lower backpressure, shorter run time and sharper peaks.

2.4. UHPLC-MS analysis

UHPLC-MS analyses were performed using an Accela1250 UHPLC system (Thermo Fisher Scientific) coupled to a Thermo Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an ESI ion-source in negative ionization mode. The spray voltage was set at 3.0 kV whereas the capillary, tube lens and skimmer voltage were set to -75 V, -190 V and -46 V respectively. The capillary temperature was set at 300 °C. Sheath- and aux-gas flow rate was set at 60 and 5 units respectively. Generally, a scan range of m/z 125–2500 was used in full-MS mode and m/z 77.5–1550 for MS/MS mode. The Thermo Exactive Orbitrap mass spectrometer model used in this study lacks a quadrupole mass filter and therefore was not capable of parent ion mass filtering before ion fragmentation. As a result, all ions were fragmented together in MS/MS mode.

Compounds were separated using an Acquity UPLC CSH C18 column (2.1 × 150 mm, 1.7 μm; Waters, cat#: 186005298) coupled to an Acquity UPLC CSH VanGuard pre-column (2.1 × 5 mm, 1.7 μm; Waters, cat#: 186005303). The column assembly was maintained at 55 °C with an eluent flow rate of 300 μL/min to allow for shorter analysis times. The injection volume was 5 μL. Eluent A consisted of MQ:MeCN (40:60) containing 5 mM of ammonium formate, and eluent B consisted of MQ:MeCN:1-BuOH (0.5:10:90) also containing 5 mM ammonium formate. A linear gradient elution was applied as follows: 5 % eluent B for 2.5 min; a gradient from 5 % to 90 % eluent B over 36.5 min; holding for 3 min; returning to 5 % eluent B in 0.5 min; and holding for 8 min. The narrow

gradient window method is the same with a 45 % eluent B baseline and that the gradient runs from 45 % to 90 % eluent B over 19.5 min maintaining the same gradient slope (2.31 % B / minute).

Lipid headgroups, the total amount of carbon atoms in the ether- or acyl tails and number of double bonds therein were identified by full-MS and MS/MS using an in-house calculated *in-silico* lipid database and comparison to lipid standards of the same class where possible. Analyte retention time trends were also used as an additional indicator for identification. Lipid acyl tail isomers were identified using full-MS/MS in a similar fashion as described elsewhere (Oursel et al., 2007; Cabrera et al., 2000; Houjou et al., 2004).

3. Results

3.1. Separation of a lipid standard

To improve upon the ability of current 2-PrOH-based gradient RPLC methods to separate highly hydrophobic lipids, the eluotropic strength of the eluents was increased by using 1-BuOH instead of 2-PrOH, and the elution profile was adjusted compared to previously described methods (Wörmer et al., 2013; Damen et al., 2014). Thus, lipids were loaded on the column with eluent A consisting of MQ:MeCN (40:60) and 5 mM of ammonium formate whereas eluent B consisted of MQ:MeCN:1-BuOH (0.5:10:90) and 5 mM ammonium formate. After sample injection, a linear elution gradient was applied (See Materials and Methods). Standard solutions of lipids belonging to several different classes were injected on a CSH C18 UPLC column to establish retention times of commonly observed, well-characterized lipids and internal standards for lipid extractions. All lipid species (Table 1) were detected in the negative ionization mode predominantly as $[M-H]^-$ ions with the exception of phosphatidyl choline (PC 18:1/18:1). For this lipid, a formate adduct ($[M + CHO_2]^-$) was the most abundant ion, presumably caused by the positive charge of PC promoting formate ion attachment. The separation of these species is demonstrated in Fig. 1. Additionally, mobile phase performance was compared to the same mobile phase with 2-PrOH instead of 1-BuOH and two 2-PrOH-based mobile phases described in literature (Damen et al., 2014; Criscuolo et al., 2019) (Supplementary

Table 1

List of the compounds injected as lipid standards with peak numbering, corresponding retention time (t_R) and observed mass. The peak numbers correspond to the chromatogram in Fig. 1. AG, archaetidyl glycerol. *: Likely sn2 and sn1 lyso-lipid isomer separation respectively. **: The observed and theoretical mass of PC refers to the $[M + CHO_2]^-$ adduct as PC preferentially forms formate adducts.

Peak #	t_R	Compound	observed $[M-H]^-$	Theoretical $[M-H]^-$	ppm error
1	2.59	DDM	509.29707	509.29674	0.648
2	3.54/ 3.89*	LPG (18:1)	509.28871	509.28849	0.432
3	3.92/ 4.27*	LPA (18:1)	435.25208	435.25171	0.850
4	5.09	PG (10:0/ 10:0)	553.31512	553.31471	0.741
5	13.8	Oleic acid (18:1)	281.24881	281.24860	0.747
6	21.5	PG (18:1/ 18:1)	773.53443	773.53381	0.802
7	22.12	PA (18:1/ 18:1)	699.49795	699.49703	1.315
8	23.01	PG (18:0/ 18:1)	775.55019	775.54946	0.941
9	24.37	PC (18:1/ 18:1)	830.59298**	830.59166**	1.589
10	24.87	PE (18:1/ 18:1)	742.54022	742.53923	1.333
11	26.83	AG (20:0/ 20:0)	805.67018	805.66918	1.241
12	33.08	CL (4 × 18:1)	1456.02867	1456.02755	0.769

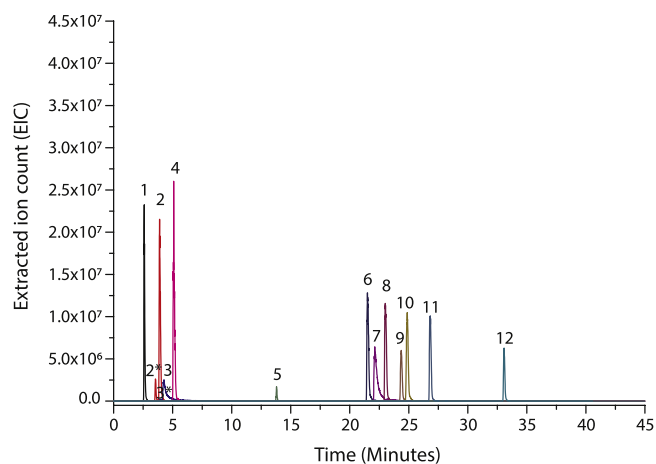


Fig. 1. LC-MS extracted ion chromatograms showing separation of defined lipid standards containing a variety of lipid classes using the full gradient method. The peak numbers correspond to the compounds listed in Table 1.

Fig. 1 and Supplementary Tables 1–3).

With the employed elution gradient slope using the 1-BuOH-based mobile phase (See Materials and Methods), all lipid species separate well and are visible as separate peaks, including some different classes with the same lipid core (i.e., di-oleoyl-ester glycerol phospholipids). In general, lipids are mainly separated based on their hydrophobicity, meaning lipids with more carbon atoms on the acyl chains generally elute later, whereas the introduction of double bonds in the acyl chain causes analytes to elute earlier. These results are consistent with previous findings using 2-PrOH-based methods (Lísa et al., 2017; Damen et al., 2014). In addition, the presence of ether bonds instead of ester bonds likely contributes to the later elution of archaeal diether lipids compared to their corresponding bacterial diester lipids. Both LPG and LPA show a smaller peak eluting just before the main peak, which can be attributed to the *sn1/sn2* acyl migration equilibrium of 90 % *sn1* and 10 % *sn2* isomer (Plückthun and Dennis, 1982).

Noteworthy, previously used RP methods often suffered from peak tailing problems with lipids containing amine and ammonium groups such as phosphatidyl ethanolamine (PE) and PC, respectively. This peak tailing is suggested to be caused by interactions between the amine and ammonium groups on the lipid headgroup with free silanol groups of the column packing (O’Gara and Wyndham, 2006). However, no significant PE or PC peak tailing was observed with either the CSH or BEH technologies of the columns employed in this work (Damen et al., 2014). On the contrary, in the lipid standard where the PE peak shows slight tailing similar to most other analytes, the PC peak shows slight fronting. PA and phosphatidyl serine (PS) often exhibited excessive peak tailing when analyzed using RPLC methods in the past as well (Ogiso et al., 2008). However, with the current method, only moderate peak tailing of PA (and LPA) was observed in the lipid standard (Fig. 1).

Overall, from the elution profile of the standard lipids, it appears that the 1-BuOH-based method effectively separates a wide range of lipids, including the more hydrophobic cardiolipin and archaeal diether lipid species. The overall retention time is reduced compared to 2-PrOH-based methods, leaving chromatographic space for the analysis of more hydrophobic compounds (Supplementary Fig. 1). However, this comes at the cost of decreased ionization efficiency of less hydrophobic lipids in the lipid standard mix using the same ionization conditions.

3.2. Separation and analysis of an *E. coli* cardiolipin extract

An *E. coli* cardiolipin extract was analyzed to validate the efficacy on a lipid mixture that is difficult to separate and strongly retains on C18 columns. For this purpose, a narrower gradient window was applied to optimize separation of more hydrophobic analytes (see Materials and

Methods). The MS was set to negative mode while alternating between full-MS mode and CID MS/MS (80 eV) each scan. The lipid mixture eluted in 5 peak clusters (Fig. 2A). However, a cardiolipin database mass search revealed the presence of a 6th cluster containing very small amounts of lipids not visible in the TIC chromatogram (Supplementary Table 4). In addition, the mobile phase performance was compared to the same mobile phase with 2-PrOH instead of 1-BuOH and two previously published 2-PrOH-based mobile phases (Damen et al., 2014; Criscuolo et al., 2019) (Supplementary Fig. 2 and Supplementary Tables 1–2).

In order to investigate the effect of small differences in acyl tail composition, the mass of CL 16:0/16:1/16:0/16:0 and of closely related CLs (CL molecules with a single different acyl chain) were extracted from the total ion chromatogram and plotted in Fig. 2B. This revealed that the cluster elution interval corresponds to the loss or gain of a double bond or a C₂H₄ moiety (Fig. 2B and Supplementary Fig. 3). The peaks in a cluster were incompletely separated based on other features such as the presence of cyclopropyl groups or, for example, double bond isomers. Noteworthy, the separation observed here is similar to that of positional isomers as reported previously for PC diester lipids (Nakanishi et al., 2010). However, no positional isomers were identified with sufficient confidence to confirm the capability to separate acyl positional isomers of CL. The separation data collected does allow for a calculation of approximate retention time shifts for a given modification of a base lipid. For example, CL 16:0/16:1/16:1/16:0 (*t_R* 14.71) elutes 0.86 min earlier, CL 16:0/16:1/Cy17:0/16:0 (*t_R* 15.32) elutes 0.25 min earlier

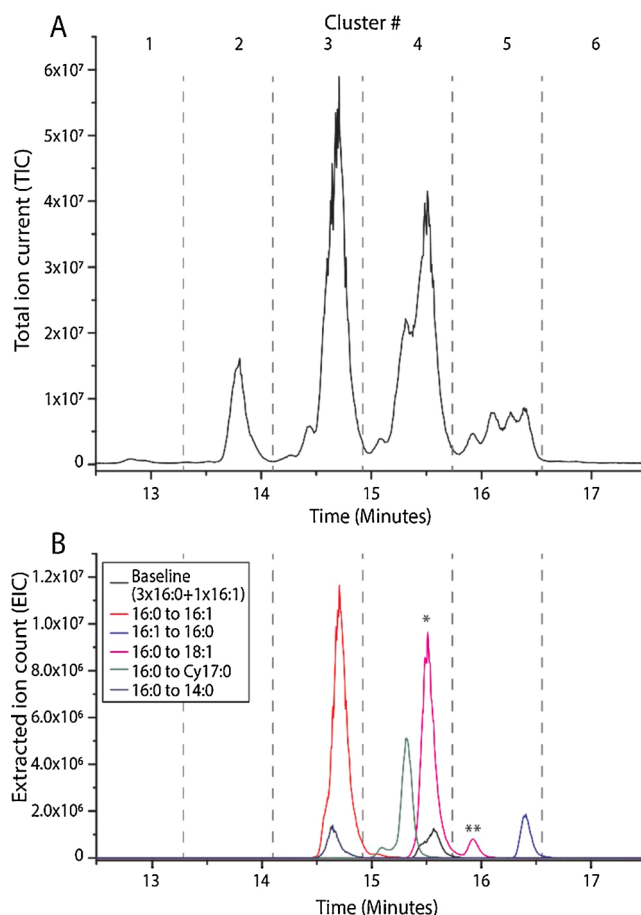


Fig. 2. LC-MS chromatograms showing (A) the elution of various cardiolipin species in 6 peak clusters in a total ion chromatogram using the narrow gradient window method. (B) Extracted ion chromatograms of selected CL molecule masses that are closely related to CL 16:0/16:1/16:0/16:0, differing by a single acyl substitution as listed in the legend. Peak * represents CL 16:0/16:1/18:1/16:0 whereas peak ** represents the isomeric CL 16:0/Cy17:0/Cy17:0/16:0.

and CL 16:0/16:1/14:0/16:0 (t_R 14.64) elutes 0.93 min earlier than CL 16:0/16:1/16:0/16:0 (t_R 15.57). Thus, based on these molecules, a retention time increase of approximately ~ 0.47 min per introduced CH_2 moiety in the fatty acyl chain is observed. Alternatively, the introduction of a double bond or cyclopropyl moiety resulted in a retention time decrease of 0.86 and 0.72 min respectively. This effect is amplified when the sample is analyzed using two in-line coupled CSH UPLC columns (result not shown). As shown in Fig. 2B, some lipids elute in multiple peaks. Slightly different retention properties of different lipid modifications can cause isomeric lipid species to elute at a different time. For example, CL 16:0/Cy17:0/Cy17:0/16:0 elutes slightly later than its isomer CL 16:0/16:1/18:1/16:0 (Fig. 2B and Supplemental Fig. 5).

The MS/MS spectra yielded strong signals of acyl carboxylate ions allowing for easy identification of acyl chain moieties. As a result of ketene and fatty acid elimination, $[\text{M}-\text{H}-\text{RCHCO}_{sn1/2}]^-$ and $[\text{M}-\text{H}-\text{RCH}_2\text{COOH}_{sn1/2}-\text{H}_2\text{O}]^-$ ions were also detected. Due to preferred elimination of the $sn2$ over the $sn1$ ketene/fatty acid moiety, the MS/MS data was used to determine the acyl chain substituent position of several of the more abundant cardiolipin species as originally reported for *Lactobacillus acidophilus* and *E. coli* diester lipids (Oursel et al., 2007; Cabrera et al., 2000; Houjou et al., 2004) (Supplementary Table 4, Supplementary Figs. 4 and 5). Using this method, our analysis showed that there is a preference for 16:0 acyl chains on the outer chain positions ($sn1$) while there is a preference for alternative chain substituents on the inner chain positions ($sn2$) of cardiolipin molecules present in this *E. coli* cardiolipin extract. Overall, this analysis demonstrates that this method is capable of differentiating between similar and large lipids containing more than 50 carbon atoms in the lipid tails. In comparison with conventional 2-PrOH-based mobile phases, our method showed improved separation of these hydrophobic lipid species. In general, a larger retention time difference between peak clusters was obtained, which resulted in the appearance of some extra total ion current peaks. Moreover, using the 1-BuOH-based mobile phase resulted in a significantly better peak shape for lyso-cardiolipins.

3.3. Separation and analysis of an *E. coli* total lipid extract

To illustrate that the RP-UHPLC method can be used for conventional full-scale lipidomics, an *E. coli* total lipid extract was injected using the full-gradient method (5–90 % B) in order to also adequately retain and separate less hydrophobic lipids. The *E. coli* lipidome was then analyzed for the presence of the major phospholipid class species. Masses corresponding to at least 33 distinct phosphatidyl glycerol (PG) and 40 distinct PE diester glycerophospholipids were identified in the extract (Supplementary Table 5). Diesters with the same core, but a different head group are well separated from one another as in the standard lipid mixture. Additionally, masses corresponding to 36 different cardiolipins are well separated from diester lipids (Fig. 3).

The wide variety of lipid species separated in this complex sample shows that the 1-BuOH-based method is versatile. At the same time it allows for very similar lipid species with only minor structural rearrangements to be separated as well. This is exemplified by the extracted ion chromatogram of one of the more abundant diester lipids, PG (33:1). This phospholipid was selected as it contains acyl chains with an even and uneven number of carbon atoms in length, where one of the acyl chains either contains a cyclopropyl moiety or a double bond. This theoretically allows for many combinations of acyl chains with slightly different properties and possibly resulting in multiple chromatographic peaks for the same mass. In a similar fashion to the cardiolipin extracted ion chromatograms (Fig. 2B), the extracted ion chromatogram of PG (33:1) reveals multiple peaks, two main peaks with the major peak showing a shoulder (Supplementary Fig. 6). The retention time differences between the peaks suggests that these are isomers containing cyclopropyl moieties and/or double bonds. The MS/MS data indicates that the major peak represents PG (16:0/Cy17:0). However, due to the lack of a mass filter in the instrument used, the acyl tail identity of the

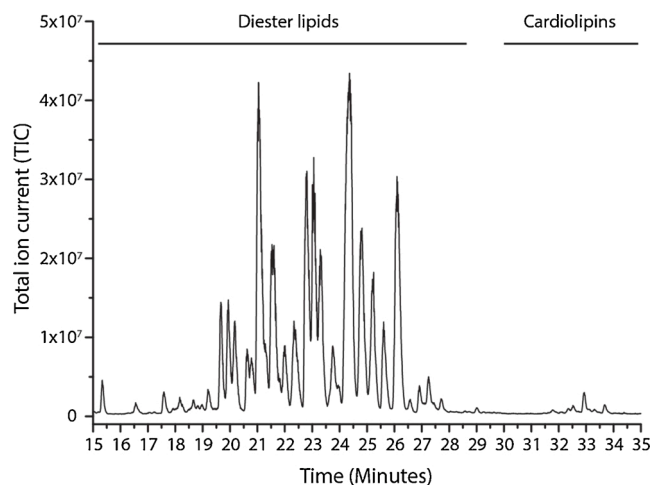


Fig. 3. A total ion LC-MS chromatogram showing the elution of an *E. coli* DH5 α total lipid extract using the full gradient method.

other isomeric PG lipid species could not be confidently established using the lyso- and acyl fragments. Multiple other co-eluting PG lipid species rendered it impossible to confidently assign specific acyl tails to the $sn1$ - or $sn2$ -position of particular parent ions.

3.4. Separation and analysis of a *S. acidocaldarius* total lipid extract

Next, our RP-UHPLC method was employed to analyze archaeal lipid extracts containing tetraether lipids. A total lipid extract of the crenarchaeon *S. acidocaldarius* MW001 was injected on the column using the same full-gradient as used for the *E. coli* lipid extract. Four major elution peaks were found with masses corresponding to an inositol phosphate dialkyl glycerol diether lipid (IP-DGD, 1) and three main glycerol dialkyl glycerol tetraether (GDGT) peaks identified as inositol phosphate-dihexose (IP-DH-GDGT, 3), inositol phosphate (IP-GDGT, 4), di-hexose (DH-GDGT, 5) and a smaller caldarchaeol (GDGT, 6) peak (Fig. 4A). It appears that *S. acidocaldarius* tetraether lipids elute later (35–40 min, Fig. 4A) with 76–88 % eluent B, as compared to the cardiolipins from *E. coli* (30–35 min, Fig. 3 and Supplementary Table 5) with 64–76 % eluent B, illustrating the separation of more hydrophobic compounds.

The addition of a hexose to a tetraether lipid containing no hexoses usually results in a significant retention time decrease (e.g. GDGT to MH-GDGT and IP-GDGT to MH-IP-GDGT, Fig. 4B). However, the retention time decrease for additional hexoses suffers from diminishing returns, resulting in a smaller retention time decrease for each hexose added. Possibly, hexose addition on the less polar glycerol backbone shields a part of the hydrophobic tetraether core from interactions with the C18 column, resulting in a reduced retention time. Thus, GDGTs with two hexoses on the same end (DH-GDGT) should have a different retention time from GDGTs with a single hexose on both ends (MH-MH-GDGT) due to a difference in lipid core shielding. In accordance with literature, we only observe a single chromatographic peak for the mass corresponding to MH-MH-GDGT and DH-GDGT indicating the presence of only DH-GDGT. MH-MH-GDGT or other tetraether lipids with two hexose headgroups have not been described for the *S. acidocaldarius* lipidome, but these lipids have for instance been detected in sediment cores taken from the Northern Arabian sea (Besseling et al., 2018; Elling et al., 2017).

Normal phase HPLC methods are particularly well suited to separate tetraethers with different numbers of cyclopentane rings in the lipid core (Schouten et al., 2007; Jensen et al., 2015a; Shimada et al., 2008; Zeng et al., 2019). Therefore, the capability of our RP-UHPLC method to separate tetraether lipids containing different amounts of rings was investigated using the narrow LC gradient (Fig. 5, Supplementary Table 6). Separation was apparent from tetraether lipid species

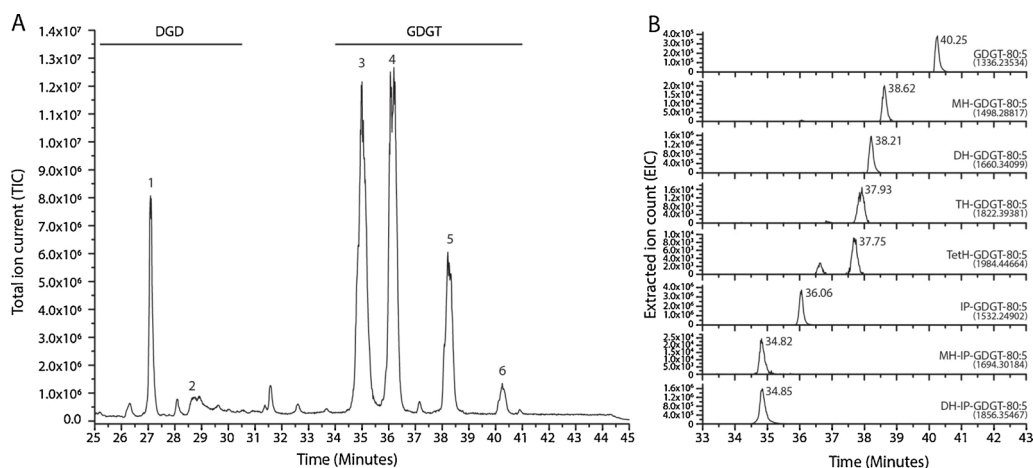


Fig. 4. (A) Total ion LC-MS chromatogram showing the elution of a *S. acidocaldarius* MW001 total lipid extract using the full gradient method. (B) Extracted ion chromatograms of GDGT-80:5 species and their +1 isotopologue containing varying numbers of hexose units. GDGT-80:5 indicates a tetraether with 80 carbon atoms in the alkyl chains containing 5 cyclopentane rings. The theoretical mass for each observed formate adduct ion ($[M + CHO_2]^-$) is listed below the name.

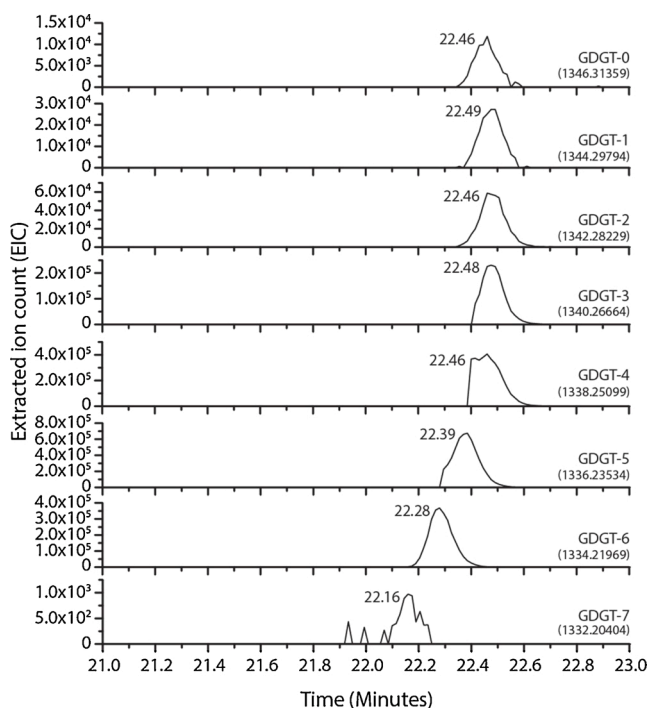


Fig. 5. Extracted ion count chromatograms of masses corresponding to formate adducts of GDGT core lipids ($[GDGT + CHO_2]^-$) with different numbers of rings from a *S. acidocaldarius* MW001 total lipid extract eluted with the narrow gradient window method. The name format used is “GDGT-X”, where “X” denotes the number of rings that lipid species has with the theoretical formate adduct ion mass for that lipid species listed below the name.

containing 5 rings or more and improved with a further increase in the number of rings. Thus, the method is capable of tetraether lipid separation of species with different numbers of rings to some extent.

4. Discussion & conclusions

4.1. 1-Butanol as a strong eluent in RPLC

Here we report on a versatile RP-UHPLC-ESI-MS method that can be used to separate and analyze complex biological lipid mixtures. The method depends on a routinely used C18 column but employs 1-BuOH

instead of 2-PrOH as a solvent in the secondary mobile phase. While different hydrophobic alcohols, including 1-BuOH, have been used in the past as organic modifiers to alter the chromatographic properties of mobile phases, 1-BuOH is currently not widely used as primary eluent in RPLC separations (Benhaim and Grushka, 2008; Lavine et al., 2002). The use of this stronger eluent facilitates earlier elution of common lipid analytes and allows for the elution of highly hydrophobic lipid species (e.g. cardiolipins, tetraethers and mycolic acids) before the end of the elution gradient, enhancing separation.

Analytical coverage is one of the barriers for further advancement in the field of lipidomics (Rustam and Reid, 2018). The use of stronger eluents, such as 1-BuOH in RPLC, pushes that barrier forward and allows for more method flexibility for the aforementioned application. Overall, RPLC methods, such as this one, are suited to analyze eukaryotic or bacterial lipidomes which often have a large diversity of acyl lipid tail species in a particular class. Although, most archaeal organisms lack the lipid tail diversity of bacteria or eukaryotes, this method is also of interest for archaeal lipidomics. As it showed an enhanced ability to elute highly hydrophobic lipid species such as tetraether lipids, which are often found in archaeal membranes.

4.2. Separation of different lipid classes

Besides the separation of strongly hydrophobic lipid species, different classes (e.g.: PG, PA, PC and PE) of less hydrophobic lipids such as lyso-, di-ester and di-ether lipids can also be separated based on their headgroup. Sufficient separation of these lipid classes reduces the chance to observe ion suppression effects and aids in detailed structural characterization. Separation effects based on lipid headgroup hydrophilicity are also apparent for highly hydrophobic species such as tetraether lipids. However, additional modifications that further increase headgroup hydrophilicity, such as adding additional hexose moieties, seem to suffer from diminishing returns. Although some GDGTs containing different numbers of cyclopentane rings could be separated, normal phase methods remain far superior for this purpose.

4.3. LPA and PA peak tailing

The peaks corresponding to LPA and PA did show moderate tailing in the lipid standard. PA is known to exhibit excessive tailing in RPLC separations and has an affinity to metals. To address this issue, Ogiso and coworkers (Ogiso et al., 2008) previously showed that an increase in buffering agent concentration, a decrease in pH 6.7 to pH 4 and the addition of 5 μ M phosphoric acid to the mobile phase, suppressed the

unidentified PA column interactions. The addition of phosphoric acid possibly resulted in more competing anion interactions from the mobile phase, thereby suppressing the unidentified PA analyte interactions, but at the same time may harm MS equipment. Additionally, the increase in buffer concentration and change of pH can cause retention changes potentially requiring method redevelopment specifically for LPA/PA analysis. Later, Cífková and coworker (Cífková et al., 2016) also described the tailing behavior of PA during HILIC method optimization and managed to reduce PS and PA tailing. This was accomplished by employing a Hydride-modified silica HILIC column (replacing Si—OH with Si—H bonds) and using a relatively high concentration (40 mM) of ammonium formate pH 4 in the mobile phase. However, with our RPLC method, LPA and PA tailing was found to be greatly attenuated when using a column with a passivating coating on the inner metal surface of the column (Supplementary Fig. 7). This suggests that phosphate-metal interactions with metal column surfaces, not only free silanol group interactions, are a major factor leading to the observed peak tailing of phosphatidic acids such as LPA and PA using the RPLC method described in this work.

4.4. Adaptations for more specific applications

Alternative techniques for the analysis of highly hydrophobic compounds exist; for example, non-aqueous reversed-phase (NARP) LC. NARPLC is often used to analyze triacylglycerol lipids, mycolic acids and other highly hydrophobic compounds (Řezanka et al., 2014; Holčápek et al., 2009; Ndlandla et al., 2016; Lisa et al., 2011; Parrish et al., 2007; Dugo et al., 2006). Typically, NARPLC uses RPLC columns but requires organic solvents that are often incompatible with RPLC system configurations. Although, the total exclusion of water from the eluents used for the 1-BuOH-based RP-UHPLC method is not possible due to buffer solubility issues; the miscibility and solvation properties of 1-BuOH enable the use of high proportions of this solvent. The high proportion of 1-BuOH and low amount of water allows the RP-UHPLC method described here to approach NARP-LC conditions and elute very hydrophobic compounds (e.g. mycolic acids) near the end of the elution gradient (Supplementary Figs. 8 and 9) (Lisa et al., 2007; Tahiri et al., 2020).

As this method can be run on RPLC configured machines, it is more widely applicable compared to either NPLC or NARPLC. Moreover, in contrast to NARP, this RPLC method is also suitable to analyze less hydrophobic compounds, highlighting its versatility. Eluent A was designed to allow for separation of less hydrophobic analytes such as fatty acids and lyso-lipids during method development. Combined with the eluotropic strength of 1-BuOH in eluent B, this resulted in a single method capable of separating different analytes ranging from bacterial fatty acids to highly hydrophobic cardiolipins, archaeal tetraethers or methoxy mycolic acid in a single run; making this a useful versatile LC separation method for lipid research. Moreover, the pronounced selectivity towards the acyl chain in bacterial lipids from the C18 column also allows this method to be used for detailed acyl chain composition analysis per lipid class.

4.5. Potential improvements

Although the 1-BuOH-based method described here has a larger analytical range compared to conventional mobile phases, the instrument response decreased for the moderately polar lipids in the lipid standard mixture compared to using 2-PrOH-based mobile phases (Supplementary Fig. 1). Possibly, the ionization efficiency decreased because 1-BuOH is less volatile compared to 2-PrOH. Different ionization conditions or an ionization technique such as heated-ESI (HESI) might be more appropriate for the use of this method for the analysis of moderately hydrophobic lipid mixtures; or possibly atmospheric pressure chemical ionization (APCI) for mixtures containing mostly highly hydrophobic lipids such as tetraether lipid cores or mycolic acids. The

use of ammonium formate as buffering agent is suitable while running negative mode ESI-MS to promote the formation of negatively charged adduct ions; especially for lipids with a positively charged headgroup such as PC or neutral glycolipids, which prefer to form formate adduct ions over deprotonation. However, other buffering agents or additives, such as formic acid, acetic acid or ammonium acetate, could be used to increase the ionization efficiency, improve the chromatographic peak shape of particular analytes, or promote positive ion formation in positive mode ESI-MS (Wörmer et al., 2013; Cajka and Fiehn, 2016).

Furthermore, the gradient used in the 1-BuOH method described in this work can readily be adjusted for particular lipid classes of interest, allowing for shorter analysis times that can at least be reduced by half. The method could potentially be further improved by substituting MeCN with methanol in the mobile phase, potentially improving peak shapes of specific analytes and GDGT response (Wörmer et al., 2013). Furthermore, if desired, it might be possible to develop a NARP or NARP-capable method based on methanol or MeCN as eluent A, against 1-BuOH as eluent B. With only eluent B or both eluents excluding water. Moreover, formic- or acetic acid instead of ammonium formate or -acetate could possibly be used to avoid buffer precipitation and promote positive ion formation. In theory, other more hydrophobic solvents, equipped with different eluotropic properties, could be used as well. However, special care must be taken to avoid issues with instrument compatibility (e.g.: pump seals, increased back pressure), mobile phase miscibility, buffer solubility, and possibly decreasing ionization efficiency.

4.6. Final remarks

1-BuOH has a low long-term health impact compared to several commonly used NPLC solvents and is also less flammable and not known to form dangerous oxidation products over time. This makes 1-BuOH more user friendly compared to many other alternative LC-MS solvents. From a sustainability point of view, 1-BuOH can be obtained from bio-renewable sources in contrast to for example, methylene chloride, tetrahydrofuran, chloroform or hexane, lowering the environmental footprint of this lipid analysis method compared to other common methods.

Data availability statement

All relevant data is available in the manuscript and accompanying supplementary material.

Authors contributions

NdK, AJM and AJMD developed the concept and designed the study. NdK, ME and RA performed the study, acquired the data and analyzed the data. RA synthesized the lipid molecules that are not commercially available. NdK drafted the article, and all authors contributed to revising it critically form important intellectual content. All authors approved the final version of the submitted manuscript.

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Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

Declaration of Competing Interest

The authors have no competing interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at <https://doi.org/10.1016/j.chemphyslip.2021.105125>.

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