A critical assessment of glyco- and phospholipid separation using silica chromatography

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Abstract

Phospholipid derived fatty acids (PLFA) are commonly used to characterize microbial communities in situ and the phylogenetic position of newly isolated microorganisms. PLFAs are obtained through separation of phospholipids from glycolipids and neutral lipids using silica column chromatography. We evaluated the performance of this separation method for the first time using direct detection of intact polar lipids (IPL) with high performance liquid chromatography-mass spectrometry (HPLC-MS). We show that the phospholipid fraction does not, under standard or modified conditions, contain only phospholipids, but also other lipid classes such as glycolipids, betaine lipids and sulfoquinovosyldiacylglycerols. Thus, commonly reported PLFA compositions likely are not purely derived from phospholipids and perhaps may not be representative of fatty acids present in living microbes.

1. Introduction

Examining microbial communities in situ is one of the major challenges in microbial ecology. Traditionally, isolation and cultivation techniques were used, next to microscopic observations, to characterize microorganisms in environmental samples. This traditional approach has its limitations as it was estimated that, depending on the habitat, only about 0.001 to 1% of all microorganisms are cultivable by standard techniques (1). Over the last two decades, a number of cultivation-independent techniques for the examination of microbial community composition have been established, using in particular genomic techniques.

One of the earliest alternative approaches to study microbial communities independent of cultivation was the analysis of the composition and abundance of fatty acids in environmental samples and comparison to the fatty acid composition of pure cultures (2-4). Fatty acids do...
not occur as such in living biomass but as intact polar lipids (IPL) with the fatty acids esterified via either a glycerol or amide moiety to a polar head group. These fatty acids are released after cell death and can persist in natural environments. Therefore, fatty acids derived from living biomass have to be distinguished from those derived from dead and fossil biomass when analysing microbial communities in situ. One way to do this is to study phospholipid derived fatty acids (PLFA) as phospholipids are thought to be relatively labile and fall apart shortly after cell death (5, 6). Furthermore, they are the major lipids in cell membranes, and are rarely used as storage products. In contrast, glycolipids are generally classified as storage products (7), although it was recently shown, that they can also be major membrane lipid components in chloroplasts of plants, algae and in cyanobacteria (8). PLFAs are thus commonly used as indicators for living microbes. The interpretation of the PLFA patterns of environmental samples is done by comparison with the PLFA patterns of microorganisms grown in pure cultures (9-11). Furthermore, they are used to investigate metabolic activity in situ by stable isotope analysis in combination with labelling experiments (12, 13). PLFA analysis is also often used as chemotaxonomic information to characterize the phylogenetic position of new microorganisms (14).

In order to obtain PLFAs rather than free fatty acids or fatty acids contained in storage lipids, Vorbeck and Marinetti (15) proposed a method to separate bacterial lipids in a ‘neutral lipid’, ‘glycolipid’ and a ‘phospholipid’ fraction by applying a silicic acid column and eluting the fractions with different mixtures of chloroform, acetone and methanol (MeOH). The obtained phospholipid fraction was subsequently hydrolysed to obtain phospholipid-derived fatty acids which could be analysed by gas chromatography. The efficacy of the silicic acid column separation was verified by measuring the phosphorus and the carbohydrate content in the different fractions using colorimetric methods (carbohydrates by the anthrone reaction after Radin et al. (16) and phosphorus content by the molybdenum blue method after Harris
and Popat (17) and modified by Marinetti et al. (18)). The separation method has subsequently over the years been modified by decreasing the volumes of eluents used, replacing chloroform with the less toxic dichloromethane (DCM) and the use of pure acetone and methanol, to obtain the neutral, glyco- and phospholipid fractions, respectively (9, 19-21). This modified separation method is now routinely used in environmental studies as well as for chemotaxonomy of microbes belonging to the bacteria and eukaryotes (10, 22-24). However, after the initial studies, the performance of the separations has been rarely re-evaluated, particularly whether the PLFAs are truly derived from phospholipids only. Additionally, recent studies have shown, that both betaine lipids and sulfoquinovosyldiacylglycerols (SQDG) are abundant IPLs in the marine environment (25-29). The fate of these IPLs after separation on a silica column is, to the best of our knowledge, unknown and thus it is unclear to what degree the fatty acids contained in these membrane lipids are accounted for in PLFA analysis.

In the last 15 years analytical techniques were developed that made it possible to directly analyse IPLs, including phospholipids, using high performance liquid chromatography mass spectrometry (HPLC-MS). Typically, IPLs are separated according to the polarity of the headgroup and identified by multistage mass spectrometry (11, 30, 31). However, this type of analysis does not allow detailed identification of the various fatty acids contained in the IPLs.

For detailed identification, separation and hydrolysis of the lipid extract and subsequent GC(MS) analysis is still required. Additionally, for stable isotope probing, which requires GC-amenable compounds, PLFAs are still needed. Therefore, PLFA analysis is still an important method within microbial ecology.

In this study we have re-assessed the composition of the different chromatographic fractions isolated by silica chromatography, using lipid extracts from different environmental samples. Through direct analysis of IPLs using HPLC-MS we accurately studied the fate of.
several environmentally important types of IPLs during one of the most used chromatographic fractionations and assessed whether PLFAs are truly representative of membrane lipids.

2. Material and Methods

2.1. Sampling

A marine sediment was collected in January 2012 during low tide in the Mokbaai on the Dutch Wadden Island Texel. A microbial mat sediment was collected in August 2010 on the Dutch Wadden Island Schiermonnikoog. Similar microbial mats from the same location were described by Bolhuis and Stal (32). The microbial mat was sampled from the top of the sediment and immediately sealed. Sediment cores with a diameter of 7 cm were taken by hand, sliced on location and the first cm was collected. All samples were freeze dried, homogenized at stored at -40 °C until extraction.

2.2. Extraction of intact polar lipids

The freeze dried samples were extracted using a modified Bligh-Dyer method (11, 33). In short, the samples were extracted with MeOH/DCM/Phosphate buffer 2/1/0.8 (v/v/v) ultrasonically three times for 10 min. The supernatants were collected to which DCM and phosphate-buffer were added to achieve a phase separation. The DCM fraction was transferred to a round bottom flask and the aqueous phase was washed three times with DCM. All DCM fractions were combined and dried using a rotary evaporator. The resulting Bligh Dyer Extract (BDE) was transferred into a vial with DCM/MeOH 9:1, further dried under a nitrogen flow and stored dry at -20°C.
2.3. Separation of different IPL-classes

The BDE was split into three equal, by volume, aliquots. All subsequent treatments were performed in triplicate. In order to obtain the so-called neutral, glyco- and phospholipid fractions the BDE was separated on a DCM pre-rinsed silica column (0.5 g; activated for 3 h at 150 °C) eluting with 7 mL DCM, 7 mL acetone and 15 mL MeOH, respectively (9). The resulting fractions were dried under nitrogen and stored at -20 °C.

In order to test the effect of slightly different solvents mixtures the BDE was also separated on a pre-washed silica column (0.5 g) by eluting with 7 mL of DCM, 7 mL of acetone/MeOH (99/1) and 15 mL MeOH, respectively. These fractions were also dried under nitrogen and stored at -20 °C.

2.4. IPL analysis

For the IPL analysis, the original BDE and the different chromatographic fractions were dissolved and filtered in 250 μL of injection solvent (hexane/IPA/H2O 718/271/10). IPLs were analysed directly afterwards on a high performance liquid chromatography electrospray ionization tandem mass spectrometry (HPLC/ESI-MS²) after Sturt et al. (30) with some modifications. We used an Agilent 1200 series LC (Agilent, San Jose, CA), which was equipped with a thermostatted auto-injector and a column oven and coupled to a Thermo LTQ XL linear ion trap with an Ion Max source with electrospray ionization (ESI) probe (Thermo Scientific, Waltham, MA). To each sample 5 μg/mL of 1-O-hexadecyl-2-acetoyl-sn-glycero-3-phosphocholine (C16 PAF) standard was added in order to monitor the performance of the machine. Separation was achieved on a Lichrosphere diol column (250 x 2.1 mm, 5 μm particles: Alltech Associates Inc., Deerfield, IL) maintained at 30 °C. The following elution program was used with a flow rate of 0.2 mL/min: 100% A for 1 min, followed by a linear gradient to 66% A: 34% B in 17 min, maintained for 12 min, followed by a linear gradient to
35% A: 65% B in 15 min, where A: hexane/2-propanol/formic acid/14.8 M NH$_3$aq (79/20/0.12/0.04 [v/v/v/v]) and B: 2-propanol/H$_2$O/formic acid/14.8 M NH$_3$aq (88/10/0.12/0.04 [v/v/v/v]). The total run time was 60 min with a re-equilibration period of 20 min in between runs. The settings for the ESI were: capillary temperature 275 °C, sheath gas (N2) pressure 25 arbitrary units (AU), auxiliary gas (N2) pressure 15 AU, sweep gas (N2) pressure 20 AU, spray voltage 4.5 kV. The lipid extract was analysed by positive-ion scanning ($m/z$ 400 to 2000), which was followed by a data-dependent MS$^2$ experiment where the four most abundant masses in the mass spectrum were fragmented (normalized collision energy 25; isolation width 5.0; activation Q 0.175).

For each IPL class, the individual IPL species were identified in the total BDE by their fragmentation pattern in MS$^2$ (34). In order to evaluate the chromatographic behaviour of the different IPL classes during silica chromatography, the three to five most abundant IPL species within each IPL class were selected for quantification. The peak area of these IPL species were integrated in their MS$^1$ mass chromatograms. Since the same equivalent amount of extract was consistently injected, the peak areas of the total extract and the different fractions could be directly compared. To calculate the distribution of the IPL’s over the fractions, the peak areas of the selected species within an IPL class in each fraction were summed and the percentage relative to the total amount in both the acetone and the methanol fraction was calculated. The final percentage is the average of the percentage in each of the triplicates. To determine the recovery, the peak areas of the selected species within an IPL class in each fraction was summed up and the percentage relative to the amount in the BDE could be calculated as identical injection volumes and concentrations were used for the fractions and BDE, respectively. The final value is an average of the triplicates. Due to different ionization efficiencies of different IPL classes, the different treatments for each IPL...
class were compared and not the amount of IPL classes with each other within a single fraction.

3. Results and Discussion

3.1. IPL composition

To evaluate the commonly used separation method in PLFA analysis, we subjected extracts from a microbial mat (Schiemmonnikoog, the Netherlands) and a marine sediment (Mokbaai, the Netherlands) to the various silica chromatography protocols.

We first studied the IPL composition of the whole extract of both samples prior to chromatographic separation (Fig. 1). This showed that the microbial mat sediment contained two types of glycolipids, monogalactosyldiacylglycerols (MGDG) and digalactosyldiacylglycerols (DGDG) (see Fig. 2 for structures). For both of these IPLs classes, the main sugar moiety has been recognized as galactose which is why they are in general referred to as galacto-lipids (27). Both of these glycolipids mainly contained C\textsubscript{16} and C\textsubscript{18} fatty acids with 0-3 double bounds in different combinations (Table 1). In addition to glycolipids, the microbial mat sediment also contained three different phospholipid classes, phosphatidylglyceride (PG), phosphatidylethanolamines (PE) (Figs. 1 and 2). The different phospholipid classes contained mainly two fatty acids with a combined total number of carbon atoms 30 to 40 and total amount of double bonds of 0-6 (Table 1). Due to the lack of specific fragments we were not able to assess the composition of individual fatty acids in these lipid classes. Sulfoquinovosyldiacylglycerols (SQDG) (Fig. 2), which structurally resemble glycolipids but contain a sulphate group, were also found in the extract. The SQDGs contained mainly C\textsubscript{16} and C\textsubscript{18} fatty acids with 0-3 double bounds. Lipids
with a diacylglyceryl-hydroxymethyl-trimethylalanine (DGTA) head group (Fig. 2), belonging to the class of betaine lipids, were also detected in the extract. The DGTAs contained mainly C$_{16}$ and C$_{18}$ fatty acids with 0-2 double bounds (Fig. 1 A).

The BDE extract of the Mokbaai sediment also contained MGDGs and DGDGs, both containing mainly C$_{16}$ and C$_{20}$ fatty acids with 0-5 double bounds (Table 1). Of the different phospholipid classes, both PGs and a large variety of different PCs could be identified, containing two fatty acids with a combined total carbon number of 31 to 42 and a total number of double bonds of 0-12. SQDGs were much less diverse in their fatty acid composition, containing mainly C$_{16}$ fatty acids with 0-2 double bounds (Table 1). We also found DGTAs in this sample, consisting mainly of C$_{16}$ and C$_{18}$ fatty acids with no or one double bound (Fig. 1 B).

Thus, both the microbial mat and the marine sediment contain a variety of IPLs including glycolipids (MGDG and DGDG) as well as phospholipids (PG, PC, and PE) making them suitable for evaluation of the commonly used silica separation method. In addition, they contained other IPLs such as SQDG and betaine lipids (DGTA) for which it is not known in which fraction they elute.

3.2. Standard separation method

Analysis of the different chromatographic fractions obtained from the most commonly used silica gel separation method showed for the microbial mat that MGDGs were distributed evenly between the acetone (‘glycolipid’) and the methanol (‘phospholipid’) fraction, 54±5% and 46±5%, respectively (Table 2). In contrast, all of the DGDGs were detected in the methanol fraction. Of the phospholipids only PCs eluted exclusively in the methanol fraction. Both PGs and PEs were also present in minor amounts in the acetone fraction, i.e. 9±1% and 11±1% in the acetone and 91±1% and 89±1% in the methanol fraction, respectively. Of the
‘non-phospholipids’, the majority (68±3) of the SQDGs eluted in the methanol fraction, although a relatively high percentage (32±1%) eluted in the acetone fraction. DGTAs were exclusively found in the methanol fraction (Table 2). We also assessed the overall recovery of the different IPLs by normalizing on the original amounts in the extract. Of the DGDGs, SQDGs, DGTAs, and PCs 75-98% could be recovered in the different fractions. All PGs could be recovered in the different fractions. Of all studied IPL classes the recovery of the PEs is the lowest, with 30% not recovered from the column (Fig. 3 A). The recovery of the MGDGs is higher than 100%, which at first instance may be surprising. However, this could be due to ion suppression, i.e. the ionization of the early eluting MGDGs might be suppressed due to matrix effects in the complex total BDE extract. This ion suppression may be much less in the cleaner chromatographic fractions leading to enhanced ionization and thus an increase in peak areas.

The distributions of the IPL classes over the different chromatographic fractions for the Mokbaai sediment extract were similar to those observed for the microbial mat (Table 2). The majority (61±5%) of the MGDGs eluted in the acetone fraction versus 39±5% in the methanol fraction. The DGDGs eluted nearly completely (96±5%) in the methanol fraction, with the remainder (4±5%) eluting in the acetone fraction. The PC and PGs eluted in the methanol fraction, with a small percentage of the PGs (9±2%) eluting in the acetone fraction. For the SQDGs, 67±3% eluted in the methanol and 33±3% in the acetone fraction. All DGTAs were found in the methanol fraction (Table 2). For recovery, similar results were found as for the microbial mat sediment. For example, 90-230% of the SQDGs, MGDGs and the DGTAs were recovered after separation, while about 20% of the PGs and PCs could not be recovered. The lowest recovery was for the DGDGs with 55% (Fig. 3 B).

Our results show that, independent of sample type, a large part of the phospholipids elute in the ‘phospholipid’ fraction as expected. However, crucially a major part of the glycolipids...
also elute in this fraction and not, as anticipated, in the glycolipid fraction. Furthermore, ‘non’ phospholipids like SQDGs and DGTA also elute in the ‘phospholipid’ fraction while SQDGs are also found in the ‘glycolipid’ fraction. Both lipid classes contribute to the membrane lipid pool, especially under phosphate limitation (28, 29) and therefore have to be considered when looking at membrane lipids as biomarkers for living biomass. Neither the ‘glyco’- nor the phospholipid’ fraction thus consist of glyco- or phospholipids only. These results indicate that using this common separation method, a complete separation between glycolipid and phospholipid cannot be obtained and that the ‘PLFA’ reported are not exclusively derived from phospholipids but also from glycolipids, betaine lipids and SQDGs. Therefore, we experimented with a slightly modified elution scheme in an attempt to optimize separation.

3.3. Adaptation of separation method

To assess the possibility of obtaining better separation between glyco- and phospholipids we used a mixture of acetone and methanol (99/1,v/v) as second eluent. This slightly more polar solvent should result in a slightly different separation, with possibly more of the glycolipids, especially MGDGs, ending up in the second fraction. This modified elution scheme was tested on the microbial mat extract. Indeed, slight differences in the separation of the different lipid classes were observed. For example, the major part of the MGDGs now ended up in the acetone/methanol fraction and 35±1% in the methanol fraction (Table 2). The separation of DGDGs is the same compared to that of the standard method with all lipids eluting in the methanol fraction. As in the standard method all PCs eluted in the methanol fraction, with slightly less PGs (6±1% compared to 9±1%), but slightly more PEs in the acetone/methanol fraction (18±4% compared to 11±1%). The largest difference was observed for the SQDGs, with twice as much being found in the acetone/methanol fraction compared to the original acetone fraction, 60±8% vs. 32±1%. Concerning the DGTA, 99±2% were still found in the methanol fraction (Table 2). Compared to the standard separation, recoveries for
DGDGs, PCs, PEs and PGs were similar, but for MGDGs and SQDGs the recovery was increased from 153% to 184% respond from 75% to 120%, respectively. The apparently high recovery of SQDG, could again be due to ion suppression and therefore a suboptimal ionization of the SQDGs in the complex total BDE, similar to what is observed for the MGDGs. In contrast, the recovery of the DGTAs had decreased by 40% (Fig. 3 C).

These results show that an increase in polarity of the second eluent changes the elution pattern of some of the IPL classes, but does not result in an improved separation of glyco- and phospholipids. Further adaptations of the solvent mixture ratios are unlikely to succeed in providing a better separation as either more of the glycolipids will end up in the phospholipid fraction or phospholipids will elute in the glycolipid fraction. This is due to the fact, that the separation on a silica column is based on the polarity of the head groups. Therefore, MGDGs will always elute together with PCs and SQDGs.

3.4. Implications

Our results show that it is not possible to obtain a complete separation between phospho- and glycolipids using the commonly used silica acid chromatography separation method. Furthermore, the recovery varies between different IPL classes, with some of the phospholipids (e.g. PE) not fully recovered in the ‘phospholipid’ fraction. Thus, previous studies examining PLFAs, might have analysed fractions that also contain fatty acids derived from glycolipids, betaine lipids and to some extent SQDGs, while missing out a certain amount of phospholipids and SQDGs. In the marine environment, different IPLs can contain different fatty acids (25-27, 35), and specific fatty acids do not necessarily derive always from the same lipid class. This will give a biased view of fatty acids present in microbial communities in situ. Furthermore, PLFA analysis of microbial isolates will also give an incomplete picture of the full diversity of fatty acids derived from IPLs. Therefore, microbial
fingerprints basing on PLFA fractions are unlikely to reflect the true fatty acid pattern coming from phospholipids only. Future studies using PLFA fingerprints of environmental samples and microbial biomass have to be at least aware of this less than perfect separation and, preferably, combine this with direct analysis of IPLs. Alternatively, one can just separate a neutral and a polar fraction (using DCM and methanol) with latter containing the full suite of intact polar lipids.

4. Conclusion

Silica column chromatography results in incomplete separation of glyco- and phospholipids leading to the presence of ‘non’ phospholipids in the PLFA fraction. Therefore, studies of microbial activity using stable isotope analysis of PLFAs may be biased by fatty acids derived from glycolipids, SQDGs and betaine lipids. Because of the imperfect separation and the fact that other IPL classes such as SQDGs and betaines (and even MGDGs and DGDGs) are also part of the membrane lipids of living and active microorganisms a separation in only two fractions, a neutral fraction contain free fatty acids of dead biomass, and a polar fraction, containing all intact polar lipids, is preferable. The ‘polar lipid’ derived fatty acids can then be used to study microbial communities and their activity.

Acknowledgment

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Figure and Table legends:

Figure 1: HPLC/ESI-MS base peak chromatogram of the Bligh Dyer extract of (A) the Schiermonnikoog microbial mat and (B) the Mokbaai sediment. Monogalactosyldiacylglycerol (MGDG); digalactosyldiacylglycerol (DGDG); phosphatidylglycerol (PG); phosphatidylcholine (PC); phosphatidylethanolamine (PE); sulfoquinovosyldiacylglycerol (SQDG); G. diacylglyceryl-hydroxymethyl-trimethylalanine (DGTA). For structures of IPLs, see Figure 2.

Figure 2: Overview of head groups of analyzed intact polar lipids (IPL). A. MGDG; B. DGDG; C. PG; D. PC; E. PE; F. SQDG; G. DGTA. R₁ and R₂ represent different fatty acid moieties. For acronyms of IPLs, see Figure 1.

Figure 3: Recovery of different IPL classes after separation on a silica column of (A) + (C) the Schiermonnikoog microbial mat and (B) of the Mokbaai sediment normalized to the abundance in the original extract. For structures and acronyms of IPLs, see Figure 1 and 2.

Table 1: Fatty acid composition of different IPL classes in the BDE. For structures and acronyms of IPLs, see Figure 1 and 2.

Table 2: Elution of different IPL classes after separation of BDE on a silica column. For structures and acronyms of IPLs, see Figure 1 and 2.
A

B

Relative Abundance

Time

Relative Abundance

Time
Table 1: Fatty acid composition of different IPL classes in the BDE. For structures and acronyms of IPLs, see Figure 1 and 2.

<table>
<thead>
<tr>
<th>Lipid classes</th>
<th>Microbial mat</th>
<th>Marine sediment</th>
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<tr>
<td>MGDG</td>
<td>C14:0, C16:0-16:3, C18:0-18:4, C20:3-20:5</td>
<td>C14:0, C16:0-16:4, C17:0-17:1, C17:3, C18:1, C18:4, C20:4-20:5</td>
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<tr>
<td>DGDG</td>
<td>C14:0, C16:0-16:3, C18:3-18:4</td>
<td>C16:0-16:3, C20:4-20:5</td>
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<td>PE*</td>
<td>C32:1, C34:1-34:2</td>
<td>n.d.</td>
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<tr>
<td>SQDG</td>
<td>C14:0, C16:0-16:2, C18:0-18:3</td>
<td>C16:0-16:3, C20:4-20:5</td>
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<tr>
<td>DGTA</td>
<td>C14:0, C16:0-16:1, C18:0-18:2, C19:0-19:1, C20:5</td>
<td>C14:0-14:1, C16:0-16:1, C17:0, C18:1-18:2, C20:1</td>
</tr>
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</table>

* Combined total number of carbon atoms and total amount of double bound equivalents for both fatty acid moieties
Table 2: Elution of different IPL classes after separation of BDE on a silica column. For structures and acronyms of IPLs, see Figure 1 and 2.

<table>
<thead>
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<th>Marine sediment standard separation</th>
<th>Microbial mat modified separation</th>
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<td></td>
<td>Acetone fraction [%]</td>
<td>MeOH fraction [%]</td>
<td>Acetone fraction [%]</td>
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<tr>
<td>MGDG</td>
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