A unified protocol for the high-throughput measurement of PLFAs, NLFAs, GLFAs and sterols

Stefan Gorka, Alberto Canarini, Bruna Imai, Georg Teischinger, Sean Darcy, and Christina Kaiser

Centre for Microbiology and Environmental Systems Science, University of Vienna, Vienna, Austria
(stefan.gorka@univie.ac.at)

Summary

We present an improved protocol for the high-throughput extraction of fatty acids derived from phospho-, neutral and glycolipids. Addition of ethanol to chloroform improved the separation of neutral lipids from glycolipids via silica solid phase extraction. Sterols were collected in the same fraction as neutral lipids, where both may be measured on GC-MS with a single injection. Analysis of microbial pure culture extracts shows highly similar fatty acid profiles between the three lipid fractions. In contrast, soils where highly separated in their fatty acid profiles, which probably reflects the complex metabolic interactions in natural soils.

Rationale

Phospholipid fatty acids (PLFAs) are widely used to characterize microbial communities and quantify viable biomass. A central step in the standard extraction protocol is solid phase extraction (SPE), which fractionates lipids by their polarity[1] (**Figure 1**). Advances for higher sample throughput have been made by utilizing 96 well SPE plates[2]. With SPE, phospholipids are usually eluted with methanol, after subsequently washing the silica columns with chloroform and acetone, which in fact elute neutral and glycolipids, respectively.

However, the purity of the obtained lipid fractions has recently been questioned[3]. It has been shown that the separation of neutral and glycolipids is affected by the purity (and therefore polarity) of the used chloroform. Addition of ethanol increases the recovery of the neutral lipid fraction[4], but this has not been tested with high-throughput SPE. Furthermore, there have been attempts to include extraction of ergosterol (a sterol highly specific to fungi) in the PLFA method[5,6], but knowledge about the extraction efficiency is still lacking.

Neutral lipid fatty acids (NLFAs) are mostly collected for a single arbuscular mycorrhizal NLFA, but may also be indicative for carbon storage in fungi[7] and certain bacterial species[8]. However, whether fatty acid profiles between the lipid fractions obtained by the PLFA extraction method actually show the same taxonomic specificity is still unclear.

Here, we aimed to improve the existing high-throughput PLFA extraction protocol for simultanious extraction of PLFA, NLFA, glycolipid fatty acids (GLFA) and ergosterol. We also tested the taxonomic specificity of all obtained lipid fractions on microbial pure cultures.

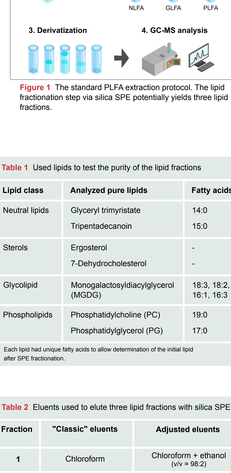

Figure 1 The standard PLFA extraction protocol. The lipid fractionation step via silica SPE potentially yields three lipid fractions.

Questions

- Does ethanol addition to chloroform improve the purity of NLFA and GLFA fractions?
- Can sterols be collected simultaneously?
- Do fatty acid profiles from the three lipid fractions show the same taxon specificity?

Methodology

We used pure lipids of different classes with distinct fatty acids to quantify their distribution in SPE fractions (**Table 1**). These standards were directly subjected to SPE, where lipid fractions were eluted with solvents of increasing polarity. Here, neutral lipids were either eluted with pure chloroform or a mix of chloroform and ethanol to test NLFA recovery (**Table 2**). Unfractionated pure lipids served as a recovery reference.

Furthermore, we analyzed fatty acid profiles of the three lipid fractions in 11 microbial pure cultures of bacterial and fungal taxa representative of the soil environment, as well as in three different soils (agricultural, grassland, and beech forest soil).

All pure culture samples were washed with ultrapure water to ensure pure biomass extracts, except for the two *Lactarius* species. Pure cultures and soils were fractionated with the adjusted eluents. Nonadecanoic acid methyl ester was added as a internal standard after fractionation for the pure culture and soil extracts. All samples were measured on a GC-TOF-MS system (LECO, USA) with a DB-5 column (Agilent, USA)

Table 1 Used lipids to test the purity of the lipid fractions

Lipid class	Analyzed pure lipids	Fatty acids
Neutral lipids	Glyceryl trimyristate	14:0
	Tripentadecanoin	15:0
Sterols	Ergosterol	-
	7-Dehydrocholesterol	-
Glycolipid	Monogalactosyldiacylglycerol (MGDG)	18:3, 18:2, 16:1, 16:3
Phospholipids	Phosphatidylcholine (PC)	19:0
	Phosphatidylglycerol (PG)	17:0

Each lipid had unique fatty acids to allow determination of the initial lipid after SPE fractionation.

Table 2 Eluents used to elute three lipid fractions with silica SPE

Fraction	"Classic" eluents	Adjusted eluents
1	Chloroform	Chloroform + ethanol (v/v = 98:2)
2	Acetone	
3	Methanol + chloroform + water (v/v/v = 5:5:1)	

"Classic" eluents after Buyer & Sasser (2012)[9]
Adjusted eluents after a suggestion in Dröber & Jeske (2019)[4]

Results

1. Lipid fractionation test

Addition of ethanol to chloroform improved the separation of NLFAs and GLFAs (**Figure 2**). The adjusted eluents achieved satisfactory fractionation of NLFAs, GLFAs and PLFAs (101%, 83% and 76% recovery, respectively). PLFA recovery could be improved to 91% by increasing the eluent volume from 0.5 to 1.5 ml (not shown). Furthermore, sterols eluted in the NLFA fraction and were unaffected by the fatty acid derivatization (117% recovery). Both NLFAs and sterols were measured with a single GC-MS injection: While fatty acid methyl esters eluted at 180-230 °C, sterols only eluted at 300 °C.

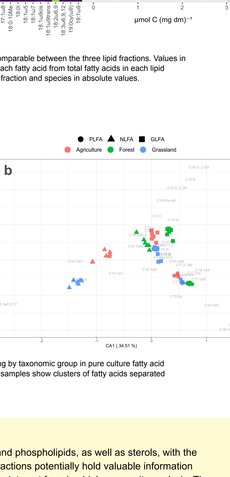

Figure 2 Fractionation with the adjusted eluents (i.e. additon of ethanol to chloroform in fraction 1) achieved an improved separation between neutral (NL) and glycolipids (GL), while leaving phospholipids (PL) unaffected.

2. Pure culture and soil extracts

We found very similar patterns between the three lipid fractions in the pure culture extracts, which indicates that taxon specificity of fatty acids is comparable between NLFA, GLFA and PLFA fractions (**Figure 3**). Fungal species showed high abundance of the established fungal markers 18:2ω6,9 and 18:1ω9cis not only in the PLFA, but also in the NLFA and GLFA fractions. Gram negative bacterial species (Proteobacteria) were strongly associated with the unsaturated fatty acid 18:1ω9trans and 19:1ω9, and Gram positive bacterial species (Actinobacteria, Firmicutes) with the terminally branched fatty acids 15:0a and 16:0i in all three lipid fractions. Fatty acid concentrations on a cellular dry mass basis were of comparable concentrations in PLFAs and NLFAs, albeit they varied strongly between species (**Figure 3**). GLFA concentrations were generally much lower than the other two fractions.

Multivariate correspondence analysis (CA) showed clustering in phylogenetic groups, regardless of the lipid fraction (**Figure 4a**). Proteobacteria and Firmicutes where strongly separated, while Actinobacteria where close to some fungal species. In contrast to the pure culture extracts, soils clustered primarily by the lipid fractions (**Figure 4b**). However, within each lipid fraction cluster, the three soil types were well separated.

Figure 3 Microbial pure culture fatty acid profiles are highly comparable between the three lipid fractions. Values in the heatmap are depicted in mol %, that is the percentage of each fatty acid from total fatty acids in each lipid fraction. The barchart shows the sum of all fatty acids per lipid fraction and species in absolute values.

Figure 4 **(a)** Correspondence analysis shows high clustering by taxonomic group in pure culture fatty acid profiles, irrespective of the lipid fraction. **(b)** In contrast, soil samples show clusters of fatty acids separated first by lipid fraction, and only secondly by soil type.

Conclusions

We were able to analyze fatty acid profiles in neutral, glyco- and phospholipids, as well as sterols, with the adjusted high-throughput extraction method. The three lipid fractions potentially hold valuable information about fungal and bacterial physiology, which is of considerable interest for microbial community analysis. The high similarities between the fatty acid profiles in the pure cultures combined with the contrasting results in soil extracts give a first understanding for future utilizations of this extraction method.

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