



Short communication

High throughput phospholipid fatty acid analysis of soils

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ABSTRACT

Phospholipid fatty acid (PLFA) analysis is widely used to measure microbial biomass and community composition in soil and other types of environmental samples. As typically performed, the analysis involves many steps and 1.5–3 days are required to prepare a small batch (*i.e.* 20–24 samples and blanks), depending on the exact equipment employed in each laboratory. Gas chromatography (GC) or gas chromatography–mass spectrometry (GC–MS) is then used to analyze the samples, requiring further time to obtain the data. We have developed a method for preparing 96 soil samples and blanks in 1.5 days, a 4- to 5-fold increase in throughput. All drying and centrifuging steps take place in a centrifugal evaporator. Soil samples in test tubes are dried overnight and then a Bligh–Dyer lipid extraction is performed. The extract is dried, dissolved in chloroform, and loaded onto a 96-well solid phase extraction plate. Phospholipids are eluted into glass vials in a 96-well format, dried, and transesterified. The resulting fatty acid methyl esters are analyzed by GC and quantified relative to an internal standard. The high throughput protocol uses much smaller solvent volumes than the traditional protocol, which combined with the use of the 96-well format leads to much faster sample preparation. Biomarker PLFA concentrations for 10 different soils were highly correlated, although not identical, between the two protocols. Multivariate analysis of the PLFA biomarkers indicated that the two protocols produced similar patterns for the different soils. The high throughput protocol may be useful to laboratories performing large numbers of PLFA analyses.

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1. Introduction

Phospholipid fatty acid (PLFA) analysis has been used to study soil microbial community responses to agricultural management (Buyer et al., 2010), heavy metals (Bååth et al., 1998), pH (Roush et al., 2009), water availability (Williams and Rice, 2007), and genetically modified crops (Blackwood and Buyer, 2004), to choose a few recent examples from a large body of the literature. PLFA analysis is somewhat slow and expensive to carry out. In our laboratory, using a recently published protocol (Buyer et al., 2010), 19 previously lyophilized soil samples plus one blank can be prepared in 1.5 days. A large-scale multifactorial experiment or environmental monitoring may require the analysis of hundreds or even thousands of samples, requiring a significant investment in time and money. In order to reduce that investment we have adapted our protocol to test tubes and 96-well plates. The use of multichannel pipettes and the 96-well plate format reduces the time required to process each sample, while miniaturization decreases evaporation time as

well as volumes of chemicals used and therefore cost and waste. These advances allow us to prepare 95 samples in 1.5 days, a 5-fold increase in throughput. In this study we present a detailed high throughput protocol and compare results to those obtained using our previous protocol, which is hereafter referred to as the standard method (Buyer et al., 2010).

2. Materials and methods

Solvents were HPLC or GC grade. Water was deionized to 16–18 MΩ cm. All other reagents were reagent grade. Bligh–Dyer extractant consisted of 200 ml 50 mM K₂HPO₄ in H₂O, 500 ml methanol, and 250 ml chloroform. The internal standard, 19:0 phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL), was dissolved in 1:1 chloroform:methanol (40.9 mg/20 ml), stored at –20 °C, and added to the extractant just before use at a rate of 0.5 μl per ml. The transesterification reagent contained 0.561 g KOH dissolved in 75 ml methanol to which 25 ml toluene was added. Glassware was cleaned as previously described (Buyer et al., 2010).

All drying and evaporation steps were performed *in vacuo* using a centrifugal evaporator (CentriVap Concentrator, Labconco, Kansas City, MO, USA). All centrifugation steps used the same centrifugal evaporator but with vacuum pump turned off.

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2.1. Drying and extraction

Approximately 2 g of moist, sieved soil was placed in previously weighed 13 mm × 100 mm screw-cap test tubes. Samples were dried *in vacuo* overnight at room temperature in the centrifugal evaporator and dry weight determined. Bligh–Dyer extractant (4.0 ml) containing internal standard was added. Tubes were sonicated in an ultrasonic cleaning bath for 10 min at room temperature before rotating end-over-end for 2 h. After centrifuging for 10 min the liquid phase was transferred to clean 13 mm × 100 mm screw-cap test tubes and 1.0 ml each of chloroform and water were added. Tubes were vortexed 5 s and centrifuged 10 min. The upper phase was removed by aspiration and discarded while the lower phase, containing the extracted lipids, was evaporated at 30 °C. Samples were stored overnight at –20 °C.

2.2. Lipid separation

Lipid classes were separated by solid phase extraction (SPE) using a 96-well SPE plate containing 50 mg of silica per well (Phenomenex, Torrance, CA, USA). Each well was conditioned with methanol (3 × 1 ml) followed by chloroform (3 × 1 ml). Samples were dissolved in 1 ml chloroform, transferred to the SPE plate, and allowed to pass through the silica. The test tube was rinsed with another 1 ml of chloroform which was transferred to the SPE plate and passed through. After washing each well with 1 ml of chloroform followed by 1 ml of acetone, phospholipids were eluted with 0.5 ml of 5:5:1 methanol:chloroform:H₂O (Findlay, 2004) into 1.5 ml glass vials (Multi-Tier microplate, E&K Scientific, Santa Clara, CA, USA). The solution was evaporated (70 °C for 30 min followed by 37 °C until dry). In order to balance the centrifugal evaporator half of the vials were moved into an extra vial rack, with alternate columns left empty in each rack.

2.3. Transesterification

Transesterification reagent (0.2 ml) was added to each vial. The vials were sealed with a PTFE/silicon cap mat (E&K Scientific, Santa Clara, CA, USA) and incubated at 37 °C for 15 min. Acetic acid (0.075 M) and chloroform (0.4 ml each) were added. After shaking for 10 s the phases were allowed to separate. The bottom 0.3 ml were removed with a multichannel pipettor into clean 1 ml vials in a Multi-Tier microplate. The chloroform extraction was repeated, this time removing the bottom 0.4 ml, and combining the extracts. The chloroform was evaporated just to dryness at room temperature. Alternate columns of vials were moved into an extra vial rack to balance the centrifuge. Samples were dissolved in 75 µl hexane, transferred to gas chromatography vials with conical glass inserts, and stored at –20 °C until analyzed.

2.4. Gas chromatography

An Agilent (Agilent Technologies, Wilmington, DE, USA) 6890 gas chromatograph (GC) equipped with autosampler, split–splitless inlet, and flame ionization detector was used. The system was controlled with MIS Sherlock® (MIDI, Inc., Newark, DE, USA) and Agilent ChemStation software. FAMES were separated on an Agilent Ultra 2 column, 25 m long × 0.2 mm internal diameter × 0.33 µm film thickness. A split ratio of 30:1 was used with hydrogen carrier gas at 1.2 ml/min constant flow rate. Initial oven temperature was 190 °C, ramping to 285 °C at 10 °C/min and then to 310 °C at 60 °C/min, followed by a hold at 310 °C for 2 min. Injector temperature was 250 °C and detector temperature was 300 °C. FAMES were identified using the MIDI PLFAD1 calibration mix and naming table.

2.5. Standard method

The standard protocol was previously described in detail (Buyer et al., 2010). Briefly, 5 g of lyophilized soil were extracted by a modified Bligh–Dyer extraction with 19 ml of extractant. After evaporation under a stream of nitrogen the lipids were separated on a solid-phase extraction column and the phospholipids eluted with 5 ml of methanol. After evaporation under nitrogen the phospholipids were transesterified to fatty acid methyl esters, extracted into 4 ml of hexane, evaporated, and analyzed by GC.

2.6. Soils

Ten different soils were run by the standard method in triplicate along with three blanks, using the internal standard described in this paper. The same 10 soils were run by the high throughput method, replicating each soil 9 times and including 6 blanks. Soil properties are presented in Table 1. While these soils represent only two of the 12 soil orders (Inceptisols and Ultisols), they do provide a range of pH, organic matter, and soil texture values. Soils 5 and 10 were taken from two locations at the Maryland Agricultural Experiment Station, Clarksville, MD, USA, while all remaining soils were collected from various locations at the Beltsville Agricultural Research Center, Beltsville, MD, USA. All soils were passed through a 4 mm screen and stored at –20 °C.

2.7. Statistical analysis

Fatty acids were summed into biomarker groups: eukaryotes, polyunsaturated fatty acids (Zelles, 1999); eubacteria, 15:0, 17:0 cyclo, 19:0 cyclo, 15:1 iso, 17:1 iso, 17:1 anteiso (Frostegård and Bååth, 1996); Gram positive bacteria, iso and anteiso saturated branched fatty acids (Zelles, 1999); Gram negative bacteria, monounsaturated fatty acids and cyclopropyl 17:0 and 19:0 (Zelles, 1999); actinobacteria, 10-methyl fatty acids (Zelles, 1999); fungi, 18:2 ω6 cis (Frostegård and Bååth, 1996); and protozoa, 20:3 and

Table 1
Soils used in this study.

Soil	Site	Soil taxonomy	pH	% Organic matter	Texture
1	Grass	Mixed Arenic Paleudults and Aquic Hapludults	4.8	0.6	Sandy loam
2	Tilled	Mixed Fluvaquentic Dystrudepts and Fluvaquentic Endoaquepts	5.1	1.4	Loam
3	Corn	Mixed Typic and Aquid Hapludults	4.7	2.3	Sand
4	Forest	Mixed Arenic Paleudults and Aquic Hapludults	4.6	2.7	Sandy loam
5	Grass	Fine-loamy, mixed, semiactive, mesic Typic Hapludults	6.5	3.1	Sandy clay
6	Grass	Mixed Arenic Paleudults and Aquic Hapludults	4.1	3.2	Sandy loam
7	Forest	Mixed Arenic Paleudults and Aquic Hapludults	4.3	3.3	Sandy clay loam
8	Grass	Fine-silty, mixed, active, mesic Aquic Hapludults	6.1	4.3	Silt loam
9	Grass	Mixed Fluvaquentic Dystrudepts and Fluvaquentic Endoaquepts	5.1	5.0	Loamy sand
10	Forest	Fine-loamy, mixed, semiactive, mesic Typic Hapludults	6.7	5.2	Sandy clay loam

Table 2PLFA analysis of variance. Differences in means between standard and high throughput method were significant ($P < 0.05$) in all cases except eubacteria.

Biomarker	Standard method			High Throughput method		
	Mean (nmol/g) ^a	SD ^b	CV (%) ^c	Mean (nmol/g)	SD	CV (%)
Total PLFA	129.42	27.33	21.12	99.59	16.59	16.66
Eukaryotes	7.90	2.61	33.04	4.95	0.37	7.47
Eubacteria	13.76	2.80	20.35	13.99	2.83	20.23
Gram-positive	32.31	7.07	21.88	20.78	3.10	14.92
Gram-negative	44.75	11.74	26.23	38.80	7.77	20.03
Actinobacteria	15.45	2.27	14.69	10.64	2.08	19.55
Fungi	4.55	2.27	49.89	2.44	0.19	7.79
Protozoa	0.87	0.34	39.08	0.55	0.13	23.64

^a Least-square means.^b Standard deviation among replicates within a soil, pooled over all soils.^c Coefficient of variance.

20:4 fatty acids (Ringelberg et al., 1997). These biomarkers are not entirely specific for their taxonomic groups and therefore must be interpreted cautiously (Zelles, 1997).

All univariate statistical analyses were conducted using SAS version 9.2 (SAS Institute Inc., Cary, NC, USA). Mixed effects analysis of variance models with log-likelihood ratio test were fit to confirm statistical similarity (among all soils) of within-method variances before pooling (across all soils) to obtain a separate estimate of within-method variability for each method. These within-method variability estimates were reported as standard deviation and %CV; statistics most appropriate to estimate the precision of each method. Multivariate analysis and ordination was accomplished using redundancy analysis in CANOCO version 4.5 (Microcomputer Power, Ithaca, NY, USA). The biomarkers for Gram-positive bacteria, Gram-negative bacteria, actinobacteria, fungi, and protozoa were Hellinger (square root of the proportion) transformed as previously described (Buyer et al., 2010). Each soil type (1–10) was binary coded and used as an environmental variable.

3. Results and discussion

Our goal was to develop a new high throughput protocol that would be significantly faster yet give results similar to the standard protocol. We therefore chose to maintain the standard Bligh–Dyer extraction chemistry (Bligh and Dyer, 1959), silica SPE for purification of phospholipids (Zelles and Bai, 1993), and base-catalyzed transesterification (White et al., 1979), while miniaturizing the assay and using the 96-well format where possible. The 96-well SPE plate enabled much higher sample throughput than running the SPE in columns. Solvent consumption was greatly reduced, and the smaller quantity of eluate speeded evaporation as well. As previously reported by Findlay (2004), we found that eluting phospholipids with 5:5:1 methanol:chloroform:H₂O required far less volume than eluting with methanol, and 0.5 ml was sufficient to achieve maximum yield. While a batch of 19 lyophilized soil samples plus 1 blank took 1.5 days to prepare for gas chromatography via the standard method, 90 soil samples dried *in vacuo* plus 6 blanks took 1.5 days to prepare with the high throughput method. For this study we analyzed the samples by GC, using a method with a run time of 11.9 min per sample, but the high throughput protocol could be used with any GC or GC–MS method.

Quantitative results comparing the 2 methods across all 10 soils are given in Table 2. The high throughput method produced lower concentrations of PLFA than the standard method but was more reproducible, as indicated by the lower standard deviations and coefficients of variation with the high throughput method. The PLFA concentrations determined by each method were highly correlated across all soils. The Pearson correlation coefficients between the two methods were 0.94 or greater for total PLFA and all

biomarkers other than protozoa, and statistically significant with $P < 0.0001$, while the protozoan biomarker had a correlation coefficient of 0.59 ($P = 0.071$). The poor correlation for protozoa may be explained by the low concentration and high variance of the protozoan biomarker (Table 2). Indeed, 9 samples had no detectable protozoan biomarker, while all other biomarkers were detected in every sample. While one could not mix the two methods in a single study, the high correlations indicate that, when comparing soils or treatments within a study, either method would lead to generally similar conclusions. The eubacteria biomarker behaved somewhat differently from all of the other biomarkers, giving similar concentrations when measured by either method. If one were to calculate eubacteria:eukaryote ratios, or any other ratio using the eubacteria biomarker, the high throughput method would give a higher ratio than the standard method. However, if two soils were compared, the soil with the higher ratio using the standard method would still have the higher ratio using the high throughput method.

It is not readily apparent why the high throughput method gives lower biomass concentrations. It may be that the extraction is somewhat less efficient in the high throughput method, although the two extraction protocols are essentially identically except for scaled down volumes in the high throughput method. Alternatively, since all concentrations were based on the internal standard, perhaps the elution of the internal standard relative to other phospholipids is higher in the high throughput method which uses a

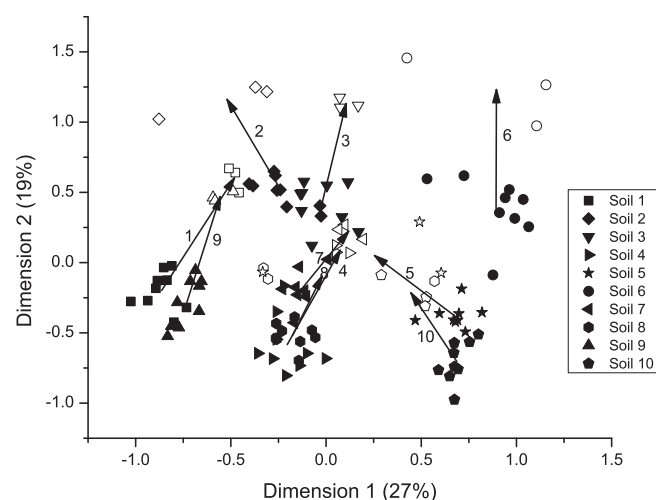


Fig. 1. Redundancy analysis of PLFA community composition. Solid symbols, high-throughput method; open symbols, standard method. Vectors represent the shift in means of each soil from the high-throughput method to the standard method. Each vector is labeled by soil number. Numbers in parentheses in axis labels represent the percent variance explained by each axis.

different solvent mixture for elution. A difference in volume of methanol used for elution was reported to change the elution profile (Mills and Goldhaber, 2010), so a difference in solvent mixture could reasonably be expected to do the same.

Redundancy analysis was used to visualize community composition among the 10 soils by both methods (Fig. 1). Both methods produced similar patterns for the different soils, although with the new method the points were shifted toward the bottom of the graph. The vectors, which point from the mean coordinates of the samples run with the new method to the mean coordinates of the samples run with the old method, show that there are some differences in how the two methods compare for the various soils, particularly in Dimension 1. However, the mean coordinates are highly correlated between the two methods (Dimension 1, $r = 0.90$, $P = 0.0004$; Dimension 2, $r = 0.97$, $P < 0.0001$). Using either method we would reach similar conclusions about the relationships between these soils: soils 5 and 10 are similar to each other, soils 4, 7, and 8 are similar to each other, and soils 1 and 9 are similar to each other. We would also conclude, using either method, that soil 2 is most dissimilar to soils 5, 6 and 10, while soil 6 is most dissimilar to soils 1, 2, and 9. Thus the two methods produce similar ecological conclusions across the range of soils run in this study. We also ran a separate redundancy analysis using both soil and method as environmental variables. While soil explained 69% of the variance in PLFA composition, method explained 17% of the variance. The difference between methods, while significant, was much less than the difference between soils.

4. Conclusions

A new sample processing method for soil PLFA analysis uses smaller samples, smaller volumes of solvents, and 96-well solid phase extraction plates. These modifications provide much higher throughput and may be useful for laboratories handling large numbers of samples. The new high throughput method does not give identical biomass results or community composition when compared to a standard method, so results cannot be combined within a single study. However, biomass results are highly correlated between the two methods, and results from ordination of community data are similar, indicating that the new method may be

expected to give similar ecological conclusions when compared to the more traditional method.

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