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Short communication

High throughput phospholipid fatty acid analysis of soils

Jeffrey S. Buyer[a,*], Myron Sasser[b]

[a] USDA, ARS, Sustainable Agricultural Systems Laboratory, Beltsville, MD, United States
[b] MIDI, Inc., Newark, DE, United States

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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 (Rousk 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 K_2HPO_4 in H_2O, 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.

* Corresponding author at: USDA, ARS, SASL, Bldg. 001 Room 140 BARC-W, Beltsville, MD 20705-2350, United States. Tel.: +1 301 504 8436; fax: +1 301 504 8370.
E-mail address: jeffrey.buyer@ars.usda.gov (J.S. Buyer).

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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:H_2O (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