SOP	Total lipid extraction from pure isolates, tissues, and environmental sample		

1. Scope

This SOP describes a total lipid extraction method using modified Bligh-dyer method. The protocol is optimized for soil samples and can be used with pure isolates of different soil organisms. The amount of extraction solvent described here can be further improved according to the freeze-dried biomass available.

2. Principle

Soluble compounds are extracted from the samples and hydrophobic compounds are separated from hydrophilic compounds by phase separation. The lower organic phase which contain most of the lipid fraction are then loaded into and LC MS system to identify and quantify the compounds.

3. References

The method is based on Buyer and Sasser (2012), with modifications derived from Dr. Yolima Carrilo (WSU).

Buyer, J. S., & Sasser, M. (2012). High throughput phospholipid fatty acid analysis of soils. Applied Soil Ecology, 61, 127-130.

Frostegård Å, Tunlid A, Bååth E. 1991. Microbial biomass measured as total lipid phosphate in soils of different organic content. Journal of Microbiological Methods 14(3): 151-163.

Kaiser C, Frank A, Wild B, Koranda M, Richter A. 2010a. Negligible contribution from roots to soil-borne phospholipid fatty acid fungal biomarkers 18:2 omega 6,9 and 18:1 omega 9. Soil Biology & Biochemistry 42(9): 1650-1652.

Schnecker J, Wild B, Fuchslueger L, Richter A. 2012. A field method to store samples from temperate mountain grassland soils for analysis of phospholipid fatty acids. Soil Biology and Biochemistry 51(0): 81-83.

4. Chemicals

Chemicals	Read and observe the chemical-related safety regulations (MSDS, Material Safety Data Sheet) before starting to work!		
	Milli-Q water	Freshly generated by Millipore device	

	Citric acid monohydrate	Riedel- de Haën 33114	
	Sodium hydroxide 50%	J. T. Baker 7067	
	Chloroform	Sigma-Aldrich 34854	
	Methanol	Sigma-Aldrich 34885	
	Ethanol (only for washing)	denatured, 96% Nr.?	
	Extrane (only for washing)	Merck MA01 107555	
	Acetone (only for washing)	technical, 99% Nr.?	
	Acetone	Fluka 00570	

5. General working instructions

Gloves	In order to prevent contamination of the samples, unpowdered lab gloves have to be worn at all steps of the protocol. Since many organic solvents are used, **nitrile gloves** are recommended.
Safety	Use the **fume hood** whenever working with organic solvents. Collect the solvent waste according to lab safety regulations.
Pipettes	Use pipettes with **Pasteur glass pipettes** as tips unless indicated otherwise (muffle Pasteur pipettes as described below). You should use PFA pipette tips if possible, when plastic is necessary. Repeatedly draw up the organic solvents to saturate the internal airspace of the pipette with the respective solvent in order to assure a precise pipetting. The maximum volume of Pasteur pipettes is **2 ml**!
Glassware	Glassware should be used throughout the procedure wherever possible.

	If necessary (e.g. for used extraction vials), soak glassware in diluted extrane solution and clean it with tap water until no stains are left. Clean all glassware in the dishwasher let it dry and cover all openings with thick aluminum foil. For extraction vials, cover a group of vials completely in foil. Put Pasteur pipettes into a large beaker and cover the opening with foil. **Muffle all glassware (packed in foil) in the furnace at 500°C for 4 hours** and let it cool down before use. Store with foil until use.
Caps and septa	Plastic caps and septa are washed intensively before use: Soak them in diluted **extrane solution** to remove traces of lipids and rinse them with tap water until no foam is visible any more. Rinse caps and septa with de-ionized water, then with ethanol and acetone. Let them dry before use. When closing vials, make sure the Teflon seals of the septa are on the inside. Do not muffle caps and septa! They will melt in the furnace.
Chemicals	Chemicals of highest purity available should be used throughout the protocol. For washing of caps and septa, chemicals of lower quality can be applied.

6. Sample preparation

Sampling	Remove living roots after sampling.	
Sample storage	(1) If possible, fresh samples should be used. (2) Alternatively, samples can be freeze-dried.	(2) Freeze the samples immediately and freeze-dry before extraction. In this case, the **water content** of the samples can be assumed zero.

	(3) It is also possible to extract frozen samples directly (although freeze-drying should be preferred). In this case, samples must not thaw during storage or transport and have to be extracted in frozen state.	(3) If continuous freezing of the samples cannot be guaranteed (e.g. when working in remote field sites), samples can be stored in RNAlater. In this case, use the SOP modified for RNAlater storage
Water content	Determine the water content of all samples before extraction.	For freeze-dried samples, the water content can be set zero.

---------- **Day 1** ----------

Soil preparation

Weigh soil	Weigh around 2 g of freeze-dried soil into **10 ml round bottom vials**. Each well plate fits 90 samples – allow space for 3 blanks and 3 repeats to check for consistency.	Use a permanent marker to label, and make sure it does not dissolve during the process!

Lipid Extraction

Citrate buffer	Prepare a 0.15 M citrate buffer. Adjust the pH to 4.0 with NaOH (liquid, 50 %).	e.g. 17.237 g of citric acid monohydrate for 500 ml buffer. This amount is sufficient for the CMB and phase separation (day 2).
CMB	- 200 ml citrate buffer - 500 ml methanol - 250 ml chloroform Mix **fresh daily**, or at least weekly if many runs are anticipated C:M:B (1:2:0.8)	950 ml is sufficient for a batch of 96 samples.
PLFA-dispenser	Before using PLFA-dispenser, rinse it with chloroform.	
Extraction	Add 6 ml of CMB to 10 ml round bottom vials that contains your soil.	Calibrate the PLFA-dispenser with Milli-Q, and note the weight of three volumes. The volume here is important, but not crucial, because this volume of extractant will not get saturated.
Sonication	Cap samples and sonicate for 10 min in sonic bath. Vortex for 30 s.	Make sure the sonicator can maintain room temperature, otherwise add ice.
	Incubate overnight at room temperature in the dark (or at least for 6 hours).	

---------- **Day 2** ----------

Phase separation

Centrifuge	Centrifuge samples and blanks at 3500 rpm for 10 min. Transfer the supernatant into new into **10 ml flat bottom vials** *or* **40 ml glass vials.**	
Re-extraction	Re-extract the pellet with 2.6 ml CMB. Vortex for 30 s and centrifuge again. Transfer the supernatant into the same new 10ml glass vial.	
Phase separation	Add 1.5 ml chloroform and 1.5 ml of citrate buffer. Vortex for 30 s. Allow overnight separation in the dark at room temperature.	Calibrate the PLFA-dispenser with MQ, and note the weight of three volumes. For the citrate buffer, the brown PLFA-dispenser can be used.

---------- **Day 3** ----------

	Label and weigh empty **10 ml flat bottom vials with caps**. Note the weight.	
	Transfer the lower (lipid) phase to the pre-weighed 10 ml vials. Take as much of the phase as possible, but make sure not to transfer anything of the upper phase.	It is not necessary to transfer all of the lower phase (see below).
	Weigh the full flat bottom vials and note the weight.	By weighing the transferred liquid, it is possible to account for losses.
N_2-drying	Rinse the needles of the N_2-dryer first with ethanol, then with acetone.	
	Dry the extract at room temperature under a constant stream of N_2. Immediately after drying, close the vials.	Drying under N_2 prevents the oxidation of fatty acids.
Storage	Dried samples can be stored frozen at -20 °C until further processing.	