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Soil quality — Determination of soil microbial diversity —

Part 2: Method by phospholipid fatty acid analysis (PLFA) using the simple PLFA extraction method

Qualité du sol — Détermination de la diversité microbienne du sol —

Partie 2: Méthode par analyse des acides gras phospholipidiques (PLFA) en utilisant la méthode simple d'extraction des PLFA

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Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 444, *Environmental characterization of solid matrices*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

This second edition cancels and replaces the first edition (ISO/TS 29843-2:2011), which has been technically revised.

The main changes compared to the previous edition are as follows:

— addition of specification for qualitative and quantitative analysis of PLFAs;

— use of BAME (qualitative) or FAME (quantitative) standards;

— use of GC-MS apparatus;

— precisions in 7.2 and 7.3;

— possibility to use commercial cartridges in addition to, or replacement of, home-made cartridges;

— update of bibliographic references.

A list of all the parts in the ISO/TS 29843 series can be found on the ISO website.

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

Phospholipids are essential components of membranes of all living cells. Extracted from soil samples in fatty acid form (phospholipid fatty acids, PLFA) or ether-linked isoprenoid side chains (phospholipid ether lipid, PLEL), they provide quantitative and qualitative insights into the soil's viable/active microbial biomass. Cellular enzymes hydrolyse and release the phosphate group within minutes to hours following cell death[1]. The determination of total PLFA and PLEL provides a quantitative measure of the viable biomass of soil, i.e. microorganisms of all three primary domains of the biosphere (bacteria, archaea and microeukaryota). PLFA and PLEL can also allow for rough taxonomic differentiation within complex microbial communities[2],[3]. Each microbial species contains several fatty acids, with a total composition in PLFA subject to the environmental conditions[4]. The approach is performed to evaluate biomass and shifts in microbial community composition[5], in what regards dominance of main groups of organisms[6]. Furthermore, combined with the use of isotope (^{13}C or ^{14}C) labelled substrates, the lipid methods can also be used to identify the metabolically active part of the microbial community. This approach is well established in soil ecology and serves as a phenotypic, and thus complementary, tool to genotyping approaches for determining microbial diversity. Apart from taxonomic descriptions, the PLFA technique enables the determination of physiological changes within microbial consortia. For instance, the monoenic PLFA 16:1ω7c and 18:1ω7c are increasingly converted to the cyclopropyl fatty acids cy17:0 and cy19:0 in Gram-negative bacteria in response to environmental stress[7].

Different methodologies are available for the determination of soil fatty acids. These methodologies present different levels of complexity when applied and provide different levels of resolution in the description of soil microbial communities. ISO/TS 29843-1 deals with the generally called “extended PLFA extraction method” while this document deals with the generally called “simple PLFA extraction method”[8],[9].

This document is accessible to most research and analytical laboratories involved in soil sciences. This methodology can be used for a wide range of soils. It provides a broad diversity measurement of a soil microbial community at the phenotypic level. It can be applied to biomass estimation and can be used to differentiate microbial communities among different soil samples (with the aid of an adapted statistical method). This method is especially adapted for detecting rapid changes in the soil microbial community structure. It can also be used to give a rough description of microbial groups present in soil samples (e.g. Gram-positive bacteria, actinomycetes, fungi[6]). Table 1 (adapted from Table 1 in Reference [8]), presents a comparison of the sensitivity of the “extended PLFA” versus “simple PLFA” techniques.

Table 1 — Comparison of the sensitivity of the “simple” and “extended” PLFA techniques in characterizing shifts in the composition of microbial communities

Property	PLFA (simple)	PLFA (extended)
Ability to differentiate between two communities (with the aid of multivariate statistical methods)	Yes	Yes
Applicability for biomass estimation	Yes	Yes
Ability to register all single components of an entire community structure (“fingerprint”)	No	Yes
Ability to register FAs other than EL-FAs	No	Yes
Estimation of number of FAs in soil samples	<50	200 to 400
Capacity to determine the linkage of the FAs to lipids in the molecule	Yes, EL	Yes, EL, NEL
Capacity to detect defined FAs in lower concentrations in the soil extract	No	Yes
Capacity to detect unusual FAs in the soil extract	No	Yes
Number of available signatures of FAs for defined organisms	Few	High numbers
Relationships of FAs widespread in the profile	High	Natura

Table 1 *(continued)*

Property	PLFA (simple)	PLFA (extended)
Ability to identify the organisms causing the shift in microbial community	No	Basically yes
FA fatty acid EL ester-linked NEL non-ester–linked		

This method has been derived from the one first proposed in Reference [10]. This revised method has been widely used[11] and has also been discussed and compared to the extended PLFA extraction method in peer-reviewed articles[8],[9].

Soil quality — Determination of soil microbial diversity —

Part 2:
Method by phospholipid fatty acid analysis (PLFA) using the simple PLFA extraction method

1 Scope

This document specifies a simple method for the extraction of only phospholipid fatty acids (PLFA) from soils.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 18400-206, *Soil quality — Sampling — Part 206: Collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

3 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

4 Symbols and abbreviated terms (except chemical products and reagents)

BAME	bacterial acid methyl ester(s)
FA	fatty acid
EL-FA	ester-linked fatty acid
NEL-FA	non-ester-linked fatty acid
FAME	fatty acid methyl ester(s)
PL-FAME	phospholipid fatty acid methyl ester(s)
w_w	mass fraction of water in the soil, in grams of water per gram of dry soil (g/g)
GC	gas chromatography
HPLC	high-performance liquid chromatography

5 Principle

Lipids are extracted using the extraction procedure in Reference [7]. Lipid extracts are fractionated on neutral lipids, glycolipids and phospholipids by liquid chromatography using an SI column. Phospholipids are transformed into fatty acid methyl esters (FAME) by mild alkaline hydrolysis. The different FAMEs are measured using gas chromatography (GC). A schematic overview of the procedures is given in Figure 1.

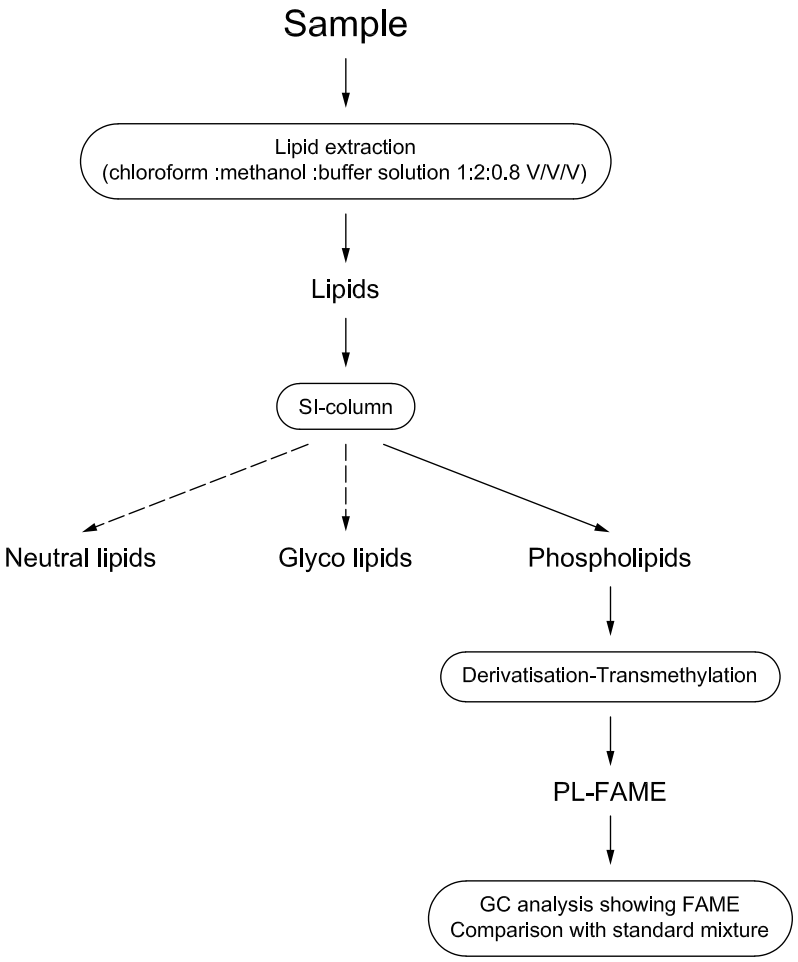

Figure 1 — Schematic overview of PLFA analysis according to the simple extraction method

6 Test materials

6.1 Soil

Collect soil samples and prepare them as specified in ISO 18400-206. Determine the soil mass fraction of water in the soil, w_w. If samples which have been sieved in the fresh state are not analysed immediately, they may be kept at –20 °C or stored in chloroform after lipid extraction (see 7.1).

6.2 Reagents

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade or HPLC grade when specified.

6.2.1 Organic solvents.

6.2.1.1 Acetone, C_3H_6O (HPLC grade).

6.2.1.2 Chloroform, $CHCl_3$ (HPLC grade).

6.2.1.3 Hexane, C_6H_{14}.

6.2.1.4 Methanol, CH_3OH (HPLC grade).

6.2.1.5 Toluene, C_7H_8.

6.2.2 Chemicals.

6.2.2.1 2,6-Di-*tert*-butyl-4-methylphenol (BHT), $C_{15}H_{24}O$.

6.2.2.2 Citric acid, $C_6H_8O_7 \cdot H_2O$.

6.2.2.3 Trisodium citrate, $C_6H_5Na_3O_7 \cdot 2H_2O$.

6.2.2.4 Silicic acid hydrate, $SiO_2 \cdot nH_2O$ if using home-made cartridges.

6.2.2.5 Anhydrous sodium sulfate, Na_2O_4S.

6.2.2.6 Potassium hydroxide, KOH.

6.2.2.7 Acetic acid, $C_2H_4O_2$.

6.2.2.8 Sodium hydroxide, NaOH.

6.2.2.9 Nonadecanoic acid methyl ester, $C_{20}H_{40}O_2$.

6.2.2.10 Nitrogen gas, N_2.

6.2.3 Buffers and standards.

6.2.3.1 Chloroform/methanol (CM) solution, to a chloroform and methanol solution with the ratio 1:2, add 2,6-di-*tert*-butyl-4-methylphenol (BHT) (0,005 %).

6.2.3.2 Citrate buffer (CB) solution, consisting of the following:

— citric acid monohydrate, 0,15 mol/l, 15,76 g of $C_6H_8O_7 \cdot H_2O$ in 500 ml of H_2O;

— trisodium citrate, 0,15 mol/l, 22,06 g of $C_6H_5Na_3O_7 \cdot 2H_2O$ in 500 ml of H_2O;

— for pH 4, add 59 ml of citric acid solution to 41 ml of trisodium citrate solution.

6.2.3.3 Bligh and Dyer (BD) solvent, to a chloroform/methanol/citrate buffer solution with the ratio 1:2:0,8, add 2,6-di-*tert*-butyl-4-methylphenol (BHT) (0,005 %).

EXAMPLE (100 ml of chloroform: 200 ml of methanol: 80 ml of CB) + BHT.

6.2.3.4 Methanolic KOH solution, 0,2 mol/l, 0,56 g of KOH in 50 ml of dry methanol (anhydrous sodium sulfate), freshly prepared.

6.2.3.5 Solvent for extraction (SE), hexane and chloroform with the ratio 4:1 (volume fraction).

6.2.3.6 Acetic acid, 1 mol/l, 58 ml/l. Add 58 ml of acetic acid to 750 ml of distilled water and fill up with distilled water to 1 l.

6.2.3.7 Sodium hydroxide, 0,3 mol/l, 12 g/l. Dissolve 12 g of sodium hydroxide in 750 ml of distilled water and fill up with distilled water to 1 l.

6.2.3.8 Standard ISTD (C19:0 FAME), 10 mg nonadecanoic acid methyl ester in 1 ml of hexane stock solution (dilution 1:100 with hexane).

6.2.3.9 Standard ESTD (BAME), Bacterial Acid Methyl Ester Mix Supelco™ #47080-U **or FAME** (ex "37 component FAME Mix" Supelco™ # CRM47885[1)], diluted 1/100 for the higher measured concentration) to perform quantification.

6.3 Apparatus

Usual laboratory equipment and the following.

6.3.1 Polytetrafluoroethylene tubes or **glass tubes**, with polytetrafluoroethylene caps or caps with polytetrafluoroethylene septum, of around 20 ml.

6.3.2 Pasteur pipettes.

6.3.3 Flasks, of capacity 40 ml, with lids with a polytetrafluoroethylene septum.

6.3.4 Glass tubes, of capacity 20 ml.

6.3.5 Home-made or commercial silica cartridges, containing 500 mg Si (as an example: SPE-Si Discovery 3 mL/500 mg, Sigma-Aldrich[2)]).

For home-made columns:

— polypropylene pipette tips (1 ml, 5 ml or 10 ml);

— ashless flocks.

6.3.6 Fume cupboard.

6.3.7 Ultrasonic bath.

6.3.8 Centrifuge.

6.3.9 Fridge.

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1) Bacterial Acid Methyl Ester Mix Supelco™ #47080-U and "37 component FAME Mix" Supelco™ # CRM47885 are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

2) SPE-Si Discovery Sigma-Aldrich silica column is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

6.3.10 Oven.

6.3.11 Vortex shaker.

6.3.12 Bain-marie equipped with a N_2 evaporation device.

6.3.13 Balance.

6.3.14 Gas chromatograph, with flame ionization detector or mass-spectrometer (for identification) with a fused silica capillary column (length 30 m minimum, internal diameter of 0,25 mm, film thickness of 0,25 µm); helium as carrier gas.

7 Procedures

7.1 Lipid extraction (Bligh-Dyer extraction)

Place 2 g of fresh soil in a 20 ml polytetrafluoroethylene tube (or an alternative, see 6.3.1). Then, add 11,9 ml of the CM solution (6.2.3.1) to the tube. Then, add CB solution (6.2.3.2) to bring the water content up to 3,16 ml. Calculate the volume of the CB solution, using Formula (1).

$$V_{CB} = 3{,}16 - \left[2 - \left(\frac{2}{1+w_w}\right)\right] \quad (1)$$

where

V_{CB} is the volume of the CB solution, in millilitres;

w_w is the mass fraction of water in the soil, in grams of water per gram of dry soil.

Mix the sample in a vortex shaker (6.3.11) and place it into the ultrasonic bath (6.3.7) for 30 min. Leave the sample in the fridge (6.3.9) at 4 °C overnight before extraction.

Shake the sample in a vortex and centrifuge it for 10 min at 1 300 *g*. Transfer the supernatant into a clean, labelled 40 ml flask (6.3.3). Then, add 5 ml of the BD solvent (6.2.3.3) to the tube containing the soil slurry. Shake the obtained mixture again in a vortex and centrifuge it for a further 10 min at 1 300 *g*. Then, transfer the supernatant to the previous 40 ml flask. Repeat this procedure twice. Finally, add 4 ml of chloroform and 4 ml of CB solution to the flasks with the supernatants to separate the phase. Leave the flask overnight in the fridge (6.3.9) at 4 °C.

Remove and discard the upper layer (aqueous) of the sample. Dry the lower layer under nitrogen (N_2) at 50 °C. Dried extracts of the samples can then be stored for a few hours in a fridge.

7.2 Separation of lipids by SI column

To prepare home-made cartridge for lipid fractionation, activate 0,5 g of silicic acid hydrate (6.2.2.4) for 1 h at 100 °C. Put ashless flock into the 10 ml pipette tip (6.3.5). Dissolve activated silicic acid hydrate in 3 ml of chloroform (6.2.1.2) and introduce it into the tip. Allow the chloroform to dry. First, condition the home-made or commercial cartridge with 2 ml of methanol (6.2.1.4) and then 2 ml of acetone (6.2.1.1). Then, condition the cartridge with 2 ml of chloroform. It is important, from now on, not to allow the sorbent to dry out between solvents.

Reconstitute the lipid extract (from 7.1) in 300 µl of chloroform and add it to the top of the cartridge through a filter. (The filter may be made from a 5 ml pipette tip containing ashless flock and 2,5 cm of anhydrous sodium sulfate). Rinse the tube with 300 µl of chloroform and add on the cartridge.

First, add to the top of the cartridge 5 ml of chloroform, followed by 12 ml of acetone and discard the obtained eluates (containing neutral lipids and glycolipids, respectively). Allow the cartridge to dry.

Then, add 8 ml of methanol and collect the phospholipid extract in a clean, labelled 20 ml tube (6.3.4). Evaporate to dryness under N_2 at 30 °C. The obtained fraction can be stored up to three days at −20 °C until derivatization.

7.3 Derivatization — Transmethylation — Clean-up

Dissolve the fractionated sample (from 7.2) in 0,5 ml of dry methanol (6.2.1.4) and 0,5 ml of dry toluene (6.2.1.5). Then, add 1 ml of methanolic KOH solution (6.2.3.4) to perform alkaline methanolysis. Shake the sample in a vortex and incubate it at 37 °C for a minimum of 30 min.

Stop the reaction by adding 0,3 ml of acetic acid (1 M) (6.2.2.7), 5 ml of the SE solvent (6.2.3.5) and 3 ml of redistilled water. Shake and clean the sample in the ultrasonic bath (6.3.7) for 30 min and then centrifuge it at 1300 *g* for 5 min. Collect the upper (organic) layer and extract again the bottom (aqueous) layer with 2 ml of SE solvent (including steps of shaking and centrifugation at 1300 *g* for 5 min). Join the upper layer to the first one. Discard the aqueous layer.

For the final cleaning, add 3 ml of sodium hydroxide (6.2.3.7) (12 g/l) to the previous extract. This time, shake the sample for 30 s and then centrifuge it for 15 min at 1 300 *g*. Transfer the supernatant using a Pasteur pipette into a clean, labelled vial through a filter (e.g. a pipette tip with ashless flock and anhydrous sodium sulfate). Wash again the aqueous layer with 3 ml of SE solvent. Centrifuge this for 5 min at 1 300 *g* Transfer the supernatant into the same vial using the filter. Repeat these last two steps (i.e. SE solvent addition and centrifuge) and collect all three extractants in the same vial again. Evaporate to dryness under N_2 at 40 °C. Freeze the samples (–20 °C) until required for GC analysis (up to one year if some confirmations are required).

7.4 PLFA analysis

Supplement the dry sample with 50 µl of standard ISTD (6.2.3.8). Then, inject 2 µl of the sample into the capillary column (injector temperature 200 °C) of the GC (6.3.14). Use helium as the carrier gas at a flow rate of 0,8 ml/min. The oven (6.3.10) should start at 180 °C for 1 min and then the temperature should be increased to 240 °C at a rate of 2 °C per minute. The temperature should then remain constant at 240 °C for 1 min. Alternatively, other column and analytical conditions can be used to obtain appropriate PLFA separation. Identification of fatty acids is performed by comparing the results with retention times obtained from standard ESTD (6.2.3.9). The amounts of each FAME in soil extracts can be calculated as described in ISO/TS 29843-1.

NOTE According to the sensitivity of the GC detection, and for quantitative analysis of PLFAs, 450 µL hexane can be added to dilute the sample containing the standard.

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