TECHNICAL SPECIFICATION

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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 main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

In other circumstances, particularly when there is an urgent market requirement for such documents, a technical committee may decide to publish other types of document:

— an ISO Publicly Available Specification (ISO/PAS) represents an agreement between technical experts in an ISO working group and is accepted for publication if it is approved by more than 50 % of the members of the parent committee casting a vote;

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An ISO/PAS or ISO/TS is reviewed after three years in order to decide whether it will be confirmed for a further three years, revised to become an International Standard, or withdrawn. If the ISO/PAS or ISO/TS is confirmed, it is reviewed again after a further three years, at which time it must either be transformed into an International Standard or be withdrawn.

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ISO/TS 29843-2 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

ISO/TS 29843 consists of the following parts, under the general title *Soil quality — Determination of soil microbial diversity*:

— *Part 1: Method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis*

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

In this corrected version, the missing Equation (1) in 6.1 has been added.

Introduction

Phospholipids are essential components of membranes of all living cells. Extracted from soil samples in fatty acid form (PLFA: phospholipid fatty acids) or ether-linked isoprenoid side chains (PLEL: phospholipid ether lipid), 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 (Reference [1]). The determination of total PLFA and PLEL provides a quantitative measure of the viable biomass of soil, i.e. microorganisms from all three domains of the biosphere (bacteria, fungi and archaebacteria). PLFA and PLEL can also allow for taxonomic differentiation within complex microbial communities (References [2] and [3]). This approach is now well established in soil ecology and serves as a phenotypic, and thus complementary, tool to genotypic (molecular genetic) 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 (Reference [4]).

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 part of ISO/TS 29843 deals with the generally called "simple PLFA extraction method" (References [5] and[6]).

This part of ISO/TS 29843, which deals with the simple PLFA extraction method, 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). Table 1 (adapted from Table 1 in Reference [5]), 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
Ability to identify the organisms causing the shift in microbial community	No	Basically yes

This method has been derived from the one first proposed in Reference [7] and later modified in Reference [1]. This revised method has been widely used (Reference [8]) and has also been discussed and compared to the extended PLFA extraction method in peer-reviewed articles (References [5] and [6]).

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 part of ISO/TS 29843 specifies a simple method for the extraction of only phospholipid fatty acids (PLFA) from soils.

ISO/TS 29843-1 specifies an extended method for the extraction and determination of both PLFA and PLEL from soils.

2 Normative references

The following referenced documents are indispensable for the application 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 10381-6, *Soil quality — Sampling — Part 6: Guidance on the collection, handling and storage of soil under aerobic conditions for the assessment of microbiological processes, biomass and diversity in the laboratory*

ISO/TS 29843-1, *Soil quality — Determination of soil microbial diversity — Part 1: Method by phospholipid fatty acid analysis (PLFA) and phospholipid ether lipids (PLEL) analysis*

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

FAs: fatty acids

EL-FAs: ester-linked FAs

NEL-FAs: non-ester-linked FAs

FAME: 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

FID: flame ionization detector

HPLC: high-performance liquid chromatography

4 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.

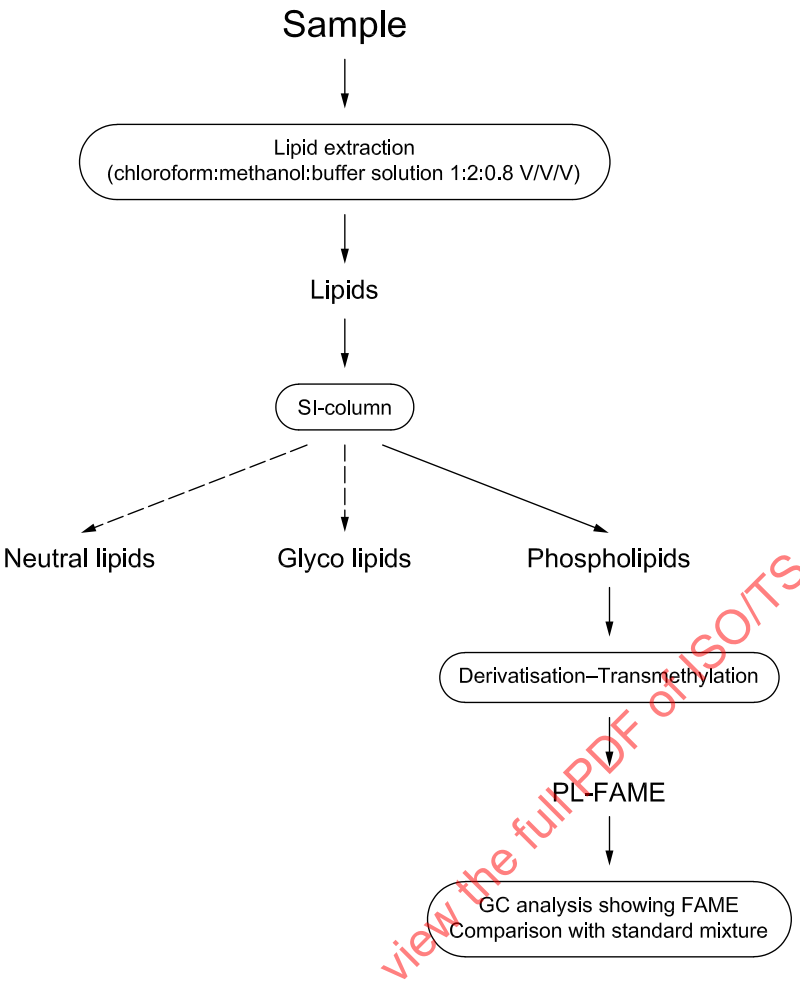

Figure 1 — Schematic overview of PLFA analysis according to the simple extraction method

5 Test materials

5.1 Soil

Collect soil samples and prepare them as specified in ISO 10381-6. 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 6.1).

5.2 Reagents

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

5.2.1 Organic solvents.

5.2.1.1 Acetone, C_3H_6O (HPLC grade).

5.2.1.2 Chloroform, $CHCl_3$ (HPLC grade).

5.2.1.3 Hexane, C_6H_{14}.

5.2.1.4 Methanol, CH_3OH (HPLC grade).

5.2.1.5 Toluene, C_7H_8.

5.2.2 Chemicals.

5.2.2.1 2,6-Di-*tert*-butyl-4-methylphenol (BHT), $C_{15}H_{24}O$.

5.2.2.2 Citric acid, $C_6H_8O_7 \cdot H_2O$.

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

5.2.2.4 Silicic acid hydrate, $SiO_2 \cdot nH_2O$.

5.2.2.5 Anhydrous sodium sulfate, Na_2O_4S.

5.2.2.6 Potassium hydroxide, KOH.

5.2.2.7 Acetic acid, $C_2H_4O_2$.

5.2.2.8 Sodium hydroxide, NaOH.

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

5.2.2.10 Nitrogen gas, N_2.

5.2.3 Buffers and standards.

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

5.2.3.2 CB (citrate buffer) 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.

5.2.3.3 BD (Bligh and Dyer) 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.

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

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

5.2.3.6 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.

5.2.3.7 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.

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

5.2.3.9 Standard ESTD (BAME), Bacterial Acid Methyl Ester Mix Supelco™ #47080-U[1)].

5.3 Apparatus

Usual laboratory equipment and the following.

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

5.3.2 Pasteur pipettes.

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

5.3.4 Glass tubes, of capacity 20 ml.

5.3.5 For home-made columns:

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

— ashless flocks.

5.3.6 Fume cupboard.

5.3.7 Ultrasonic bath.

5.3.8 Centrifuge.

5.3.9 Fridge or **freezer**.

5.3.10 Oven.

5.3.11 Vortex shaker.

5.3.12 Bain-marie.

5.3.13 Gas chromatograph, with flame ionization detector with a fused silica capillary column (length = 30 m, internal diameter = 0,25 mm, film thickness of 0,25 µm); helium as carrier gas.

1) Bacterial Acid Methyl Ester Mix Supelco™ #47080-U 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 Procedures

6.1 Lipid extraction (Bligh-Dyer extraction)

2 g of fresh soil are placed in a 20 ml polytetrafluoroethylene tube (or an alternative, see 5.3.1). Add 11,9 ml of the CM solution (5.2.3.1) to the tube. The CB solution (5.2.3.2) is then added to bring the water content up to 3,16 ml. To calculate the volume of the CB solution, use Equation (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.

The sample is then placed on a vortex shaker (5.3.11) and put into the ultrasonic bath (5.3.7) for 30 min. The sample is left in the fridge (5.3.9) overnight.

The sample is shaken and centrifuged for 10 min at 1 200 min^{-1}. The supernatant is transferred into a clean, labelled 40 ml flask (5.3.3). Add 5 ml of the BD solvent (5.2.3.3) to the tube containing the soil slurry. The obtained mixture is shaken again and centrifuged for a further 10 min at 1 200 min^{-1}. The supernatant is then transferred to the 40 ml flask and another 5 ml of the BD solvent are added. The mixture is shaken again and centrifuged for 10 min at 1 200 min^{-1}. All supernatants are put into the flask. 4 ml of chloroform and 4 ml of CB solution are then added to split the phase. The flask is left overnight in the fridge.

The upper layer of the sample is removed and discarded. The lower layer is dried under nitrogen (N_2) at 50 °C. Samples can then be stored either in a fridge or freezer.

6.2 Separation of lipids by SI column

For cartridge to lipid fractionation, activate 0,5 g of silicic acid hydrate (5.2.2.4) for 1 h at 100 °C. Put ashless flock into the 10 ml pipette tip (5.3.5). Activated silicic acid hydrate is dissolved in 3 ml of chloroform (5.2.1.2) and introduced into the tip. Allow the chloroform to dry. The cartridge is first conditioned with 2 ml of methanol (5.2.1.4), then 2 ml of acetone (5.2.1.1). The cartridge is then conditioned with 2 ml of chloroform. It is important, from now on, not to allow the sorbent to dry out between solvents.

The lipid extract is reconstituted in 300 µl of chloroform and added 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).

First add 5 ml of chloroform, then 12 ml of acetone followed by 8 ml of methanol to the lipid extract. The elutant obtained from the methanol addition is collected in a clean, labelled 20 ml tube (5.3.4). Evaporate to dryness under N_2 at 40 °C and freeze.

6.3 Derivatization — Transmethylation — Clean-up

The fractionated sample is dissolved in 0,5 ml of dry methanol (5.2.1.4) and 0,5 ml of dry toluene (5.2.1.5), and 1 ml of methanolic KOH solution (5.2.3.4) is added. Place the sample on the shaker and then incubate it at 37 °C for a minimum of 30 min.

The reaction is stopped by the addition of 0,3 ml of acetic acid (1 M) (5.2.2.7), 5 ml of the SE solvent (5.2.3.5) and 3 ml of redistilled water. The sample is shaken and cleaned in the ultrasonic bath (5.3.7) for 30 min. The sample is then centrifuged at 1 200 min^{-1} for 5 min. The aqueous phase (bottom part) is then removed and discarded.

For the final cleaning, add 3 ml of sodium hydroxide (5.2.3.7) (12 g/l). This time, the sample is shaken for 30 s and then centrifuged for 15 min at 1 200 min^{-1}. The supernatant is transferred into a clean, labelled vial through a filter (e.g. a pipette tip with ashless flock and anhydrous sodium sulfate). The aqueous layer is washed again with 3 ml of SE solvent. Centrifuge this for 5 min at 1 200 min^{-1}. Transfer the supernatant into the same vial using