SLOVENSKI STANDARD
oSIST prEN ISO 21479:2020

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Kakovost tal - Vpliv onesnaževal na floro tal - Sestava rastlinskih maščobnih kislin v listih za oceno kakovosti tal (ISO 21479:2019)

Soil quality - Determination of the effects of pollutants on soil flora - Leaf fatty acid composition of plants to assess soil quality (ISO 21479:2019)

Qualité du sol - Détermination des effets des polluants sur la flore du sol - Composition en acides gras foliaires des plantes utilisées pour évaluer la qualité du sol (ISO 21479:2019)

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Soil quality — Determination of the effects of pollutants on soil flora — Leaf fatty acid composition of plants used to assess soil quality

Qualité du sol — Détermination des effets des polluants sur la flore du sol — Composition en acides gras foliaires des plantes utilisées pour évaluer la qualité du sol

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Foreword

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For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

This document was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological characterization*.

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

Among the more than 150 ISO standards on soil quality that have been developed, less than 40 address living organisms, and among them only five address higher plants. This is despite the importance of monitoring the adverse effects of soil quality on living organisms.

One of these five standards addresses genotoxicity[1], and four of them address emergence and/or growth inhibition[2-5]. It therefore appears that these International Standards are focused either on a very specific effect (genotoxicity), or on effects great enough to induce developmental (and, therefore, visible) phenotypes (emergence or growth inhibition of young seedlings) in soils sampled in the field. Hence, more sensitive/earlier bio-indicators of the adverse effects of pollutants on plants, such as the "Omega-3 index", are needed.

The assessment of soil contaminant effects by the Omega-3 index is based on the leaf fatty acid composition of angiosperm species grown in sites of concern. The use of the Omega-3 index has proven to be appropriate for highlighting the presence of metallic and organic contaminants (herbicides, etc.) in the soils. With this aim, physical and chemical properties (pH, N/P/K content) of soils should also be determined because plant fatty acid composition may vary as a function of nutrient content[12] and pH may influence chemical compound bioavailability. It should be noted that this bio-indicator has proved to be more sensitive (i.e. responding to lower doses of contaminants) than the biometric parameters of rate of germination and biomass[6][14]. Hence, this makes it possible to gain evidence of adverse effects of soils on plants that could not be highlighted by the rate of germination or biomass. Additionally, for in situ assessment purposes, it can be difficult to observe evident effects on the rate of germination and/or biomass of plants.

It should be noted that from a practical point of view, especially with plant species harvested in the field, and in comparison with other bio-indicators, the Omega-3 index presents several advantages.

— For fatty acid analysis, only 20 mg to 50 mg of fresh leaf tissues per sample are needed. Hence, this is not destructive for plants, and there is not a problem with getting enough tissues of one species from a given area.

— Samples of plant tissues can be stored in methanol for several days at room temperature prior to analyses.

— It is not necessary to find a particular species at a site, and that a priori any species (often chosen among the most representative) can be sampled (Clause 6).

The results of a ring test performed by six individual laboratories to assess the reproducibility and the repeatability of the method are shown in Annex A. The results obtained by the same investigator with the same sample and the same measuring instrument over a short period of time are shown in Annex B.

Soil quality — Determination of the effects of pollutants on soil flora — Leaf fatty acid composition of plants used to assess soil quality

WARNING — Contaminated soils can contain unknown mixtures of toxic, mutagenic, or otherwise harmful chemicals or infectious micro-organisms. Occupational health risks can arise from dust or evaporated chemicals. Furthermore, plants might take up chemicals from the soil and safety measures should also be considered when handling the test plants.

1 Scope

This document describes a method to compare the quality of soils by determining the fatty acid composition of the leaves of plant species grown in these soils.

This method does not make it possible to determine an optimal value of the Omega-3 index and, therefore, cannot be used to determine the intrinsic quality of a soil from a specific area (regarded as homogeneous). The method can only be used to compare the quality of soils between various areas.

This method is applicable to:

— soils from contaminated sites;

— amended soils;

— soils after remediation;

— soil with waste products (e.g. slurry, manure, sludge or composts).

Alternatively, the quality of soils can be assessed by determining the Omega-3 index of *Lactuca sativa* seedlings grown in these soils under controlled conditions (i.e. phytotronic chamber) and by comparing these values to those obtained from control soils (see Annex B).

2 Normative references

There are no normative references in this document.

3 Terms, definitions and abbreviated terms

3.1 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

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

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

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

3.1.1
Omega-3 index
% C18:3/(%C18:0 + % C18:1 + % C18:2)

Note 1 to entry: The Omega-3 index has no unit.

3.2 Abbreviated terms

For the purposes of this document, the following abbreviated term applies.

FAME(s) Fatty Acid Methyl Ester(s); C16:0: palmitic acid methyl ester; C16:1: palmitoleic methyl ester; C18:0: stearic acid methyl ester; C18:1: oleic acid methyl ester; C18:2: linoleic acid methyl ester; C18:3: linolenic acid methyl ester.

4 Principle

The method is used to assess the quality of soils by determining the fatty acid composition of the leaves of angiosperm species (see Annex C and [9-14][18]) grown on these soils. After sampling leaf tissues, their fatty acid composition is determined. For this, transesterification is carried out on the foliar tissues and the fatty acid methyl esters obtained are analysed by gas chromatography. After analysis, the % C18:3 / (% C18:0 + % C18:1 + % C18:2) ratio is calculated. The lower this ratio, the higher the adverse effects on plants induced by soils is [6][9-14][18].

5 Apparatus and reagents

5.1 Apparatus

In addition to the standard laboratory equipment, the following apparatus are required.

5.1.1 Scissors to cut leaves.

5.1.2 Graduated glass pipette, to add sulfuric acid (H_2SO_4) to methanol, pipettes to dispense the mixture into glass culture tubes (1 ml/tube), and Pasteur pipettes for recovering hexane after extraction of FAMEs.

5.1.3 Glass culture tubes (e.g. 1,3 × 10 cm) with polytetrafluoroethylene seal screw caps. These culture tubes were numbered on adhesive tape (and not directly on the glass, to prevent any risk of erasing). Tubes were checked to ensure they were not chipped (in order to guarantee their leak-tight seal).

5.1.4 System (e.g. heating block) for heating the tubes to 80 °C.

5.1.5 Benchtop centrifuge for centrifuging the tubes to 200 g to 300 g and separating the aqueous phase from hexane.

5.1.6 Gas chromatograph vials with inserts and screws caps with a polytetrafluoroethylene septum.

5.1.7 Gas chromatograph equipped with a Flame Ionisation Detector (FID) and a capillary column for separating and quantifying methyl esters of fatty acids with 12 carbon atoms to 22 carbon atoms, and for each aliphatic chain length to separate the saturated, mono-, di- and tri-unsaturated esters.

Note 1 Most of the time, the studies that led to the preparation of this document were carried out using a gas chromatograph (Hewlett Packard 5890 series II or Hewlett Packard 7890A) on a Carbowax 1,2 micron, 0,53 mm diameter, 15 m long capillary column (Altech, Deerfield, IL., USA) or on a DB-WAX 1 micron, 0,53 mm diameter, 15 m long capillary column (Agilent, Santa Clara, CA., USA), helium being the carrier gas[1)].

1) This information is given for the convenience of users of this standard and does not constitute an endorsement by ISO of these products.

5.2 Reagents

5.2.1 Methanol (99 %) and sulfuric acid (H_2SO_4), components of the transesterification solution.

5.2.2 Distilled water and hexane (99 %) for extracting the FAMEs.

6 Sampling strategies

Because plant fatty acid composition can vary as a function of climatic conditions, the compared areas should share the same climatic conditions (humidity, temperature, sunlight). In addition, because the Omega-3 index is an early indicator, its measurement is not relevant when a strong visual phenotype (highly reduced biomass, high leaf chlorosis, etc.) is detected for plants having grown in one area, and not detected in another area.

Depending on the aim of the study, one or several angiosperm species can be sampled from each area of interest. For most of the studies, even if only one species can be used for the assessment of a given site (a metallurgic landfill soil for example), it is recommended to use several species (if possible three to eight). By using only one species, it is possible to serendipitously sample a highly resistant (or sensitive) species. In addition, the larger the number of species sampled, the more representative the results will be of a "soil quality" for the overall phytocoenosis. Hence, in this case, the various areas of the site are first prospected, and species to sample are chosen among the most representative examples, common to all areas to the extent possible. One leaf (or a piece of a leaf when whole leaves are too large to be entirely immerged in 1 ml of methanol/H_2SO_4, see 8.2) from four to eight individuals per species should be sampled per area. Some plant species previously successfully used to assess the soils of contaminated sites (by organic compounds and/or metals) are indicated in Annex C.

When it is not possible to sample the same species in all the areas, it remains possible to determine the Omega-3 index but, in this case: (i) all the species sampled in a given area should be present and sampled on at least one other area and (ii) all pairs of areas should share at least one species to be sampled.

Note that for the assessment of agricultural practices, the only plant species to sample is usually the only one of interest, namely the cultivated crop. When only one species is sampled, the leaf (or a piece of leaf) of 6 to 12 individuals per area is harvested.

7 Sampling of leaf tissues

The following recommendations should be followed to sample leaf tissues suitable for subsequent analysis:

— as the transesterification response involves obtaining fatty acid methyl esters from biological samples, and the presence of water leads to hydrolysis of the esters formed, the **presence of external water on the biological samples must be avoided**. Hence, if leaves are wet, before sampling, it is necessary to remove water from their surface by the use of an absorbent paper;

— do not sample leaves under hydric (drought) or biotic (pathogens) stress. Only green leaves should be harvested;

— harvest leaves on plants of similar size. Consequently, harvest of leaves from small plants in one area and leaves from tall plants in another should not be undertaken to measure the Omega-3 index (see Annex D and [12]);

— as a precautionary measure, we recommend harvesting only mature leaves and to disregard developing ones (see Annex D);

— when only a part of the leaves from a given species is sampled, harvest the same part of the leaves (the distal part for example) for all individuals;

— as a precautionary measure, it is recommended to harvest all the plants within 2 h to 3 h (see Annex D).

8 Obtaining, extraction and analyses of FAMES

8.1 Contamination control

To prevent contamination, it is necessary to avoid any contact between the solutions with plastic, parafilm or glue, etc. To ensure the absence of contaminations (e.g. protocol errors, contaminated solutions, etc.), a test should be performed before each series of analyses by following the same protocol described in 8.2 and 8.3, but without biological tissues in the culture tubes. After GC analyses, with the exception of the peak corresponding to hexane, the profile of the gas chromatogram should not display peaks.

Avoid any contact of the solutions with plastic: this recommendation does not apply to the pipette tips used for collecting the solution of methanol/H_2SO_4 (40/1) or hexane.

8.2 Obtaining and extraction of FAMES from plant leaves

Introduce the foliar tissues (approximately 1 cm × 1 cm) into the culture tubes (see 5.1.3) containing 1 ml of a solution of methanol/H_2SO_4 (40/1). Seal the tubes using a screw cap equipped with a polytetrafluoroethylene seal. Heat them for 1 h at 80 °C. With the methanol boiling at 72 °C at a pressure of 1 atmosphere, it is mandatory to avoid any evaporation so as to cause saturation vapour pressure in the tubes. It is, therefore, important that they are perfectly plugged. It is also necessary to visually check (every 5 min for 20 min, then every 10 min) that the solution of methanol/H_2SO_4 does not boil for the duration of the heating. If during the heating the contents of the tube boil, lower the tube into the ice to cool it then completely unscrew and retighten the cap. Readjust the volume, if necessary, to 1 ml by adding methanol. If the contents are still boiling afterwards, take another tube and another cap and transfer into it the contents of the defective tube. The fatty acid composition of tissues in the tubes where the solution of methanol/H_2SO_4 has (almost) totally evaporated during the heating should not be analysed.

After 1 h of heating at 80 °C, cool the tubes (e.g. put them on ice). First add 750 µl of 99 % hexane, then 1,5 ml of H_2O. Shake vigorously by hand for 20 sec. The use of a vortex should be avoided. Centrifuge the tubes at 200 g to 300 g for 5 min to 10 min to obtain two phases. Using a Pasteur pipette, transfer 200 µl to 400 µl of hexane (upper phase) into a CG vial equipped with an insert. Close the vial using a screw-opening cap equipped with a silicone septum. Collecting the lower phase should absolutely be avoided because the water irreversibly damages the column used for the gas chromatography.

Note that following this protocol, the leaf fatty acid composition does not depend on the amount of foliar tissues put in the tube (see Annex E).

8.3 Analysis of FAMES

Carry out the gas chromatography analysis with a capillary column for separating and quantifying fatty acid methyl esters with 14 carbon atoms to 22 carbon atoms, and for each aliphatic chain length for separating the saturated, mono-, di- and tri-unsaturated esters. The FAMEs are identified by comparing retention times with standard of C16:0; C16:1; C16:3 C18:0; C18:1; C18:2 and C18:3 methyl esters.

After the gas chromatography analysis (see Annex F), consider the surface of the peaks of the chromatograph corresponding to C16:0, C16:1, C16:3 (when present), C18:0, C18:1, C18:2 and C18:3. Express the results as a percentage for each FAME F_i. The percentage is calculated by dividing the

