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

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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.

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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.

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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.

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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.

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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;

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— 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

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surface S_i of the peak for the FAME F_i by the sum of the surfaces of the peaks corresponding to C16:0, C16:1, C16:3 (when present), C18:0, C18:1, C18:2 and C18:3, i.e.

$$\% F_i = 100 \times S_i/(S_{C16:0} + S_{C16:1} + S_{C16:3} + S_{C18:0} + S_{C18:1} + S_{C18:2} + S_{C18:3}) \quad (1)$$

The results of the analyses for which a contamination is suspected should be discarded. For example, when the fatty acid composition deviates too far from the standard fatty acid composition for green leaf tissues of angiosperms (see Annexes B, E, F and [6][9-14][18]).

— %C18:0, %C16:1 and %C18:1 should each be lower than 10 %;

— C16:0 and C18:2 each between 5 % and 30 %;

— C16:3 between 0 % and 30 %; and

— C18:3 higher than 40 % of the sum (%C16:0 +%C16:1 + %C16:3 + %C18:0 + %C18:1 + %C18:2 + %C18:3).

Similarly do not consider results when (an) additional peak(s) that do(es) not correspond to C16:0, C16:3, C18:0, C16:1, C18:1, C18:2 or C18:3 appear(s) in significant proportions, e.g. peak(s) displaying a surface S higher than 0,15 × ($S_{C16:0} + S_{C16:1} + S_{C16:3} + S_{C18:0} + S_{C18:1} + S_{C18:2} + S_{C18:3}$).

One or several plant species can be sampled per area. When only one plant species is analysed or when all plant species are found in all sampling areas, standard statistical procedures generally are sufficient for analysis of results. The parametric analyses (e.g. t-test) for such data assume that the data are normally distributed, that the treatments are independent and that the variance is homogenous among the various treatments. These assumptions should be tested. If the data satisfy these assumptions, results analysis may proceed. Otherwise, nonparametric tests should be used. Solely in cases of statistically significant differences between areas, a rating is attributed to the soil of each area.

When only one species is analysed, and if significant differences between the means of the Omega-3 index by area are observed, the rating of an area should be defined as the mean of the Omega-3 index on this area divided by the highest mean of the Omega-3 index measured on the whole site, all areas included.

The Omega-3 index values may depend upon the plant species analysed. Therefore, when several plant species are analysed together, standardised values of the Omega-3 index should be used. The X_p/X_{max} ratio is therefore calculated, where X_p represents the Omega-3 index measured for the individual plant p and X_{max} represents the highest Omega-3 index obtained for the individuals of the same species at a given site, all areas included.

When several plant species are analysed and all plant species were found in all sampling areas, and if significant differences are observed between the means of the X_p/X_{max} ratio by area (all individuals of all species included), the rating of an area is defined as the mean of the X_p/X_{max} on this area (all individuals of all species included) divided by the highest mean of X_p/X_{max} measured at a given site, all areas included.

Note that it remains possible to rate and to rank areas when not all plant species under study were found in all sampling areas when (i) all species sampled on a given area were present and sampled on at least one other area, and (ii) all pairs of areas shared at least one of the same sampled species. The method to calculate ratings of the areas in this case is fully described in [10] and explained in Annex G.

It should be noted that ratings are relative: for a given site, the area with the "best soil quality" is given a rating of 1, other areas are given a lower rating. In view of our previous experience[6][9-14][18], we usually distinguish the different adverse effects of soil on plants as follows: little or no effect (rating ≥0,93), medium (0,93> rating ≥0,85), high (0,85> rating ≥0,7) and very high adverse effect (0,7> rating), relative to soil of the area with a reference rating of 1.

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9 Test report

The test report should include the following information:

9.1 A reference to this document, i.e. ISO 21479

9.2 Description of the site and areas analysed

When available, a detailed map of the given site is joined to the test report. Mention area by area whether few or many plant species were present, and the most abundant ones. Note that detailed information on physical and chemical properties is helpful for the interpretation of the results (see Introduction).

9.3 Leaf sampling

— Area by area, the plant species sampled (when possible: Linnaean classification, variety, source), the number of individuals from each of these species and when appropriate, missing individual(s);

— The part of the leaves sampled (distal, proximal), and the leaves sampled (the mature leave, the oldest leaf, etc.); and

— Any specific particularity of the status of the sampled individual (developmental stage, age, size, etc.) and when appropriate description of visual damage (photographs are recommended).

9.4 Fatty acid composition

— When appropriate, missing data (broken tubes, contaminated sample, etc.);

— The fatty acid composition of each sample and the value of the % C18:3 / (% C18:0 + % C18:1 + % C18:2) ratio, its mean and standard deviation; and

— When several species are analysed, the standardised % C18:3 / (% C18:0 + % C18:1 + % C18:2) ratio, its mean and standard deviation species by species.

9.5 Conclusion

Conclusions on the relative soil quality of the various areas are drawn following analysis of results as described in 8.2 and 8.3.

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Annex A
(informative)

Results of the ring test

A.1 General

Six laboratories (noted A, B, C, D, E and F) from three European countries (France, Portugal and the United Kingdom) were involved in the ring test.

The sampling campaign was carried out on May 31, 2017 for all participants. The site under study (27 m × 19 m) was located at Léognan, near Bordeaux (France). It was divided into several areas (of 3 m × 6 m) with each area separated by 1 m. Leaf tissues were harvested from plants growing on three different areas: Area 2, Area 7 and Area 20. These areas displayed various metal content (see Table A.1) because Areas 2 and 7 had received municipal digested and dehydrated sewage sludge between 1974 and 1993 (10 t per hectare every year for Area 2 and 100 t per hectare every second year for Area 7), whereas Area 20 did not receive any sewage sludge.

Table A.1 — Total content of metal trace elements (mg/kg)

Area	Total Metal Content (mg/kg)					
	Cd	Cr	Cu	Ni	Pb	Zn
2	6,01	21,9	85,8	23,6	179	977
7	10,3	33,8	135	39	307	1790
20 (Control)	0,62	10,9	34,2	4,03	48,6	55,5

Every laboratory, A, B, C, D, E and F, synthesised, extracted and analysed FAMEs in the Laboratoire de Biogenèse Membranaire (LBM, Bordeaux). The results obtained by the various investigators in Bordeaux are indicated with letters A1, B1, C1, D1, E1 and F1, respectively.

Moreover, laboratories A, B, C, and D additionally synthesised, extracted and analysed FAMEs in their own laboratories; the corresponding results are indicated with A2, B2, C2 and D2, respectively.

Only three plant species were found in all the areas and analysed: *Plantago lanceolata*, *Hypochaeris radicata* and *Medicago sativa*. For each series (A1 to F1 and A2 to D2), laboratories independently harvested five pieces of leaf from five individuals for each species (Figures A.1 and A.2).

After fatty acid analyses, the mean of the standardised Omega-3 index values was calculated area by area, all species included.

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a) With *Plantago lanceolata*

b) With *Hypochaeris radicata*

c) With *Medicago sativa*

Key

P indicates the parts of the leaf sampled by investigators performing analyses solely at the LBM

Figure A.1 — Plant species harvested and protocol of leaf sampling for the analyses only performed at the LBM (one sample)

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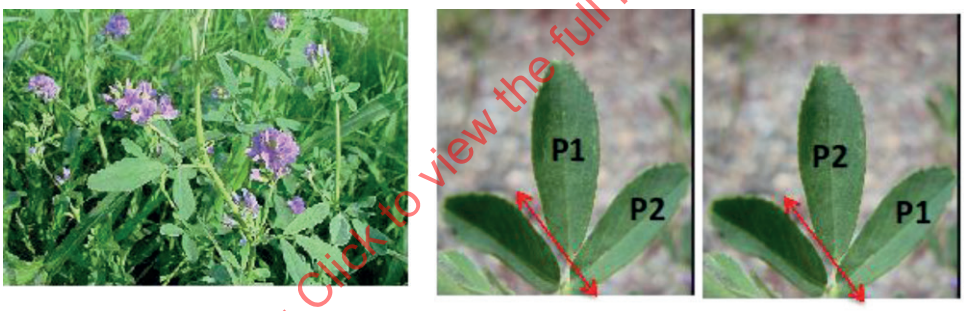
a) With *Plantago lanceolata*

b) With *Hypochaeris radicata*



c) With *Medicago sativa*

Key

P1 and P2 represent the parts of the leaf sampled by investigators who carried out analyses both at the LBM (with parts P1) and at their own laboratory (with parts P2)

Figure A.2 — Plant species harvested and protocol of leaf sampling for the analyses performed at the LBM and in the other laboratories (two samples)

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A.2 Results obtained in Bordeaux by six laboratories

Figure A.3 shows that in Areas 20 and 7, all laboratories found similar mean of standardised Omega-3 index values of the: 0,781 ± 0,016 (Kruskal-Wallis test: p = 0,883) in Area 20, and 0,786 ± 0,037 (Kruskal-Wallis test: p = 0,269) on Area 7. Green dots represent outliers.

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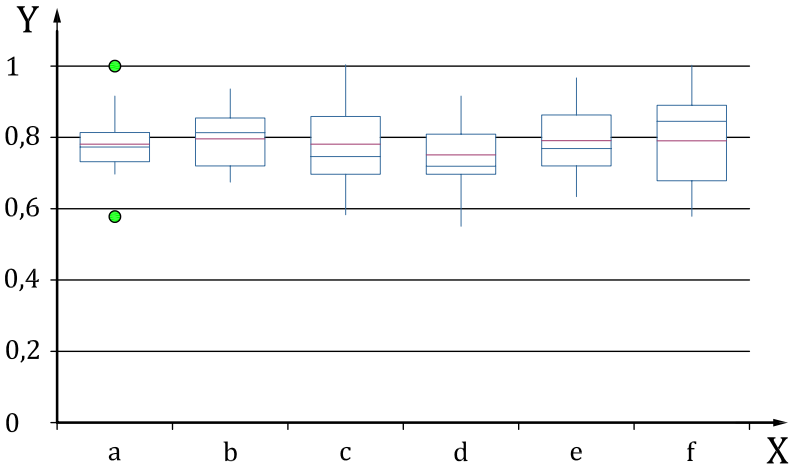
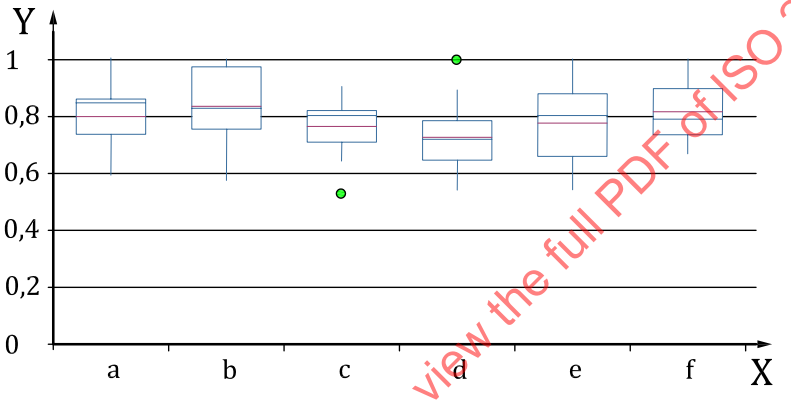


Area 20



Area 7

Key

X laboratory involved in the test

Y normalized Omega-3 index

Figure A.3 — Standardised Omega-3 Index obtained for Areas 20 and 7 for all laboratories in the LBM

By contrast in Area 2, a higher Omega-3 index, slightly but significantly higher than others, was determined for laboratory B (see Figure A.4).

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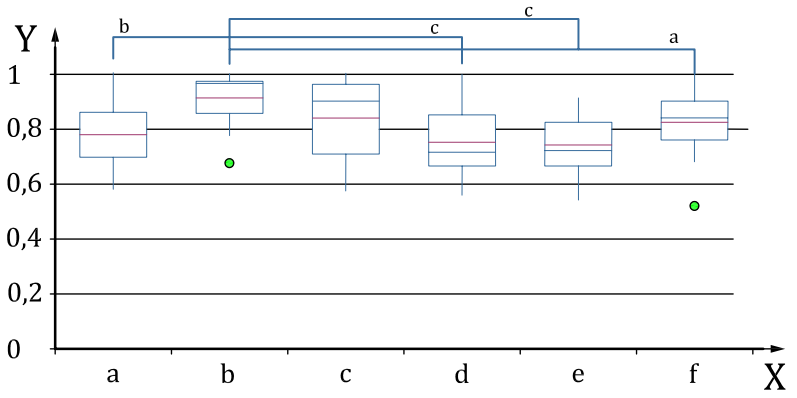

Key

X laboratory involved in the test

Y normalized Omega-3 index

Kruskal-Wallis test:

[a] $p < 0,02$

[b] $p < 0,01$

[c] $p < 0,002$

Figure A.4 — Standardised Omega-3 Index obtained for Area 2 for all laboratories in the LBM

To summarise, except for one investigator in one area (slightly higher Omega-3 index values than others), on each area, all laboratories obtained the same standardised Omega-3 index values. Because values were very close, it remains possible that among the 18 areas analysed (three areas x six laboratories), the highest and lowest values of the standardised Omega-3 index were uniformly distributed among the 17 areas, whereas one area randomly displayed a slightly greater highest value.

A.3 Results obtained by the same investigator in different laboratories:

As mentioned, laboratories A, B, C and D synthesised, extracted and analysed FAMEs from these samples in their own laboratories. Unfortunately, the laboratory D did not use a suitable GC column; therefore, it was not possible to take these results into consideration. In addition, laboratory C analysed the fatty acid composition by GC-MS and not by GC-FID in its laboratory. We discuss below the results obtained by GC-MS by laboratory C, but they cannot be directly compared to the results obtained in Bordeaux by GC-FID analyses.

Table A.2 shows that in each area, each laboratory found the lowest (orange boxes) and highest (green boxes) values associated with the same individual (all species included) in Bordeaux and in their laboratories, with the exception of one value (blue box). In addition, both of the laboratories, A and B, found similar standardised Omega-3 index values in Bordeaux (LBM) and in their own laboratories: all species and all areas included, the ratios A1/A2 and B1/B2 were 1,008 ± 0,088 (n = 45) and 1,025 ± 0,079 (n = 45) for the samples analysed by the laboratories A and B respectively. This evidence shows the low variability of the measurements when the same sample (i.e. the same leaves) was analysed in two different locations (by the same investigator).

Table A.2 — Comparisons of standardised Omega-3 Index values measured on the same leaf at LBM and the other laboratories and p-value of Kruskal-Wallis tests

Area	20				7				2			
Laboratory	A		B		A		B		A		B	
Sample N°	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2
1	0,58	0,51	0,88	0,82	0,84	0,82	0,99	0,800	0,78	0,71	0,99	1,00
2	0,73	0,62	0,92	0,88	1,00	1,00	0,82	0,77	0,83	0,89	0,97	0,89

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Table A.2 *(continued)*

Area	20				7				2			
Laboratory	A		B		A		B		A		B	
Sample N°	A1	A2	B1	B2	A1	A2	B1	B2	A1	A2	B1	B2
3	0,79	0,73	0,79	0,73	0,92	0,89	0,87	0,91	0,82	0,78	0,96	0,94
4	0,91	0,81	0,69	0,69	0,81	0,80	1,00	1,00	0,88	0,92	0,86	0,74
5	0,70	0,64	0,93	0,94	0,86	0,90	0,82	0,72	0,75	0,79	0,96	0,93
6	1,00	1,00	0,73	0,78	0,85	0,92	0,85	0,87	0,67	0,80	0,68	0,62
7	0,87	0,91	0,70	0,86	0,85	0,98	0,77	0,82	0,58	0,56	0,84	0,84
8	0,76	0,81	0,82	0,77	0,61	0,61	0,98	0,95	0,73	0,76	0,98	1,00
9	0,74	0,78	0,82	0,91	0,59	0,60	0,96	0,92	0,77	0,79	0,88	0,92
10	0,80	0,82	0,76	0,80	0,63	0,66	1,00	1,00	0,67	0,72	0,97	0,98
11	0,83	0,74	0,89	0,74	0,73	0,70	0,78	0,77	1,00	1,00	1,00	0,92
12	0,79	0,77	0,82	0,81	0,81	0,83	0,65	0,69	0,89	0,91	0,86	0,81
13	0,70	0,81	0,82	0,73	0,74	0,73	0,69	0,70	0,85	0,80	0,97	0,96
14	0,74		0,71	0,71	0,85	0,90	0,58	0,57	0,89	0,93	0,78	0,67
15	0,78	0,86	0,68	0,67	0,88	0,82	0,73	0,79	0,64	0,67	0,99	1,00
Mean	**0,780**	**0,773**	**0,798**	**0,789**	**0,798**	**0,811**	**0,832**	**0,819**	**0,783**	**0,802**	**0,913**	**0,882**
SD	0,099	0,123	0,083	0,081	0,116	0,124	0,131	0,124	0,113	0,115	0,094	0,121
C_V	0,126	0,159	0,104	0,103	0,146	0,152	0,157	0,151	0,144	0,143	0,103	0,137
p-value	***0,884***		***0,724***		***0,836***		***0,724***		***0,561***		***0,494***	

A.4 Outcome

Overall, results obtained following GC-FID analyses show that laboratories did not find any difference between areas, regardless of where the analyses were performed (except for laboratory B who found a slight, yet significant, difference between Area 2 and Area 20 for analyses conducted in Bordeaux, but not for those conducted in their laboratory). Results obtained by GC-FID and GC-MS cannot be directly compared, yet, it should be noted that GC-MS analyses (C2) did not show significant differences between areas either (Table A.3).

Looking at the total soil metal content of the various areas, it could be surprising that no differences were observed in the Omega-3 values. Nevertheless, it should be noted that metals in these areas are poorly extractable, for example 0,062, 0,187 and 0,209 mg/kg for Cd in Areas 20, 2 and 7 respectively, i.e. close to values observed in uncontaminated sites[17]. Hence, Areas 2 and 7 can be regarded as poorly contaminated, while in addition the harvested plant species are known to not be highly sensitive to metals[7,8,16].

Additionally, for any given area, the standard deviation of the normalised Omega-3 index values, all species included, obtained following GC-FID analyses by all the investigators in all the laboratories, was very low. This highlights a high reproducibility and repeatability of the results (Table A.3).

Table A.3 — Comparisons of the standardised Omega-3 Index values obtained for each area and for each laboratory and p-value of Kruskal-Wallis tests

Series	Area 20	Area 7	Area 2	*p-value* (Kruskal-Wallis)
A1	0,78 ± 0,1	0,80 ± 0,12	0,78 ± 0,11	*0,735*
B1	0,80 ± 0,08[a]	0,83 ± 0,13[ab]	0,91 ± 0,09[b]	*0,014*
C1	0,78 ± 0,12	0,77 ± 0,10	0,84 ± 0,15	*0,284*
D1	0,75 ± 0,12	0,73 ± 0,13	0,75 ± 0,15	*0,856*

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Table A.3 *(continued)*

Series	Area 20	Area 7	Area 2	*p-value* (Kruskal-Wallis)
E1	0,79 ± 0,10	0,78 ± 0,15	0,74 ± 0,09	*0,563*
F1	0,79 ± 0,13	0,81 ± 0,11	0,83 ± 0,13	*0,792*
A2	0,77 ± 0,12	0,81 ± 0,12	0,80 ± 0,11	*0,650*
B2	0,79 ± 0,08	0,82 ± 0,12	0,88 ± 0,12	*0,051*
All FID	**0,781 ± 0,014**	**0,793 ± 0,034**	**0,818 ± 0,06**	***0,277***
C2 (MS)	0,637 ± 0,107	0,674 ± 0,134	0,715 ± 0,173	*0,454*

Values of the Omega-3 index could also be analysed species by species. Since no significant differences were observed between areas (expect for B1 on Area 2), we carried out this analysis species by species, all areas included. The given coefficients of variation (C_V), in a range of 0,10 – 0,20, indicate the level of heterogeneity among the populations sampled. This heterogeneity was of the same order for all laboratories. For all the three species, the C_V values relative to the mean of the means obtained by the various laboratories were lower than 10 % (Table A.4).

Table A.4 — Comparisons of the Omega-3 Index values for the various species determined by the six investigators in the LBM

Plant species	Laboratory	A	B	C	D	E	F	Mean	SD	C_V
Plantago lanceolata	mean	3,04	3,15	3,29	3,29	3,11	3,40	3,21	0,13	0,04
	SD	0,38	0,31	0,40	0,54	0,47	0,55			
	C_V	0,12	0,10	0,12	0,16	0,15	0,16			
Medicago sativa	mean	3,75	3,69	3,63	3,85	3,54	3,62	3,68	0,11	0,03
	SD	0,42	0,60	0,53	0,60	0,57	0,51			
	C_V	0,11	0,16	0,15	0,15	0,16	0,14			
Hypochaeris radicata	mean	3,24	3,73	3,53	3,11	3,18	3,07	3,31	0,26	0,08
	SD	0,52	0,48	0,63	0,62	0,42	0,45			
	C_V	0,16	0,13	0,18	0,20	0,13	0,15			

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Annex B
(informative)

Assessment of soil quality by determining the Omega-3 index of *Lactuca sativa* seedlings grown ex situ under controlled conditions

The measurement of leaf fatty acid composition can be combined with the measurement of the effect on the early growth of lettuce seedling as described in ISO 11269-2[3]. Following various experiments and a ring test, AFNOR X 31-233[6], a standard corresponding to this method was published.

Briefly:

Cover the base of the pots with filter paper to perform the tests. Fill the pots with the soil(s) to be assessed up to approximately 0,5 cm to 1 cm below the edge. All of the pots should contain the same volume of soil not compressed and moistened to 70 % of its water-holding capacity.

Place fifteen *L. sativa* seeds per pot, distributed over the entire surface, then cover them with a fine layer of soil.

The pots corresponding to the same treatment group should be positioned randomly in the culture area.

Lettuce seedlings grow at a temperature of 18 ± 2 °C during the dark phase and at a temperature of 21 ± 2 °C during the illuminance phase, with 5 000 lx to 10 000 lx of illuminance and a 16/8 h light/darkness cycle.

Check the water evaporation by regularly weighing the pots two to three times a week. Dechlorinated water from a bubbling system is added, if necessary, until the initial weight of the pot is obtained.

One week after 50 % of the control seedlings have emerged, reduce the number of seedlings to give a total of five specimens whose biomass and developmental stage (number of leaves) are representative of the plants present in the pot.

Thirteen to 15 days after 50 % of the control seedlings have emerged, select three out of the five seedlings/pots representative of all of the seedlings of the pot.

Place the oldest leaf (i.e. the largest) of each selected seedling or, if it is too large (larger than 2 to 3 cm^2), the distal end of the oldest leaf (approximately 1 cm^2) in a culture tube containing 1 ml of a solution of methanol/H_2SO_4 (40/1). Note that the first green tissues that appear after emergence are the cotyledons (circular shape) and should under no circumstances be mistaken for leaves (oblong shape) and sampled.

The leaf fatty acid compositions are then determined as described in Clause 8. Table B.1 shows the fatty acid composition of a *L. sativa* leaf grown in control soil and determined following five consecutive analyses of the same sample by using the same GC-FID equipment.

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Table B.1 — Fatty acid composition of a *L. sativa* leaf grown in control soil and determined following five consecutive analyses of the same sample using the same GC-FID equipment

	%						Omega-3
	C16:0	**C16:1**	**C18:0**	**C18:1**	**C18:2**	**C18:3**	
	16,481	3,033	1,314	3,033	20,526	55,612	2,236
	16,517	3,103	1,301	3,003	20,420	55,656	2,251
	16,558	3,070	1,302	3,070	20,558	55,442	2,224
	16,552	3,036	1,273	3,036	20,470	55,632	2,245
	16,569	3,039	1,275	2,941	20,490	55,686	2,254
Mean	16,535	3,056	1,293	3,017	20,493	55,605	2,242
SD	0,036	0,030	0,018	0,048	0,053	0,096	0,012
C_V	0,002	0,010	0,014	0,016	0,003	0,002	0,005

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Annex C
(informative)

Plant species previously successfully used to assess soils of contaminated sites (organic and/or metals)

Family	Species	Common name
DICOTYLEDONAE		
Apiaceae	*Ombellifera sp.*	
Asteraceae	*Erigeron sp*	fleabane
Asteraceae	*Lactuca sativa*	lettuce
Asteraceae	*Lactuca seriola*	prickly lettuce, milk thistle
Boraginaceae	*Symphytum officinale*	common comfrey, common comphrey
Brassicaceae	*Brassica napus*	rapeseed
Fabaceae	*Anthylis vulneraria*	kidney vetch
Fabaceae	*Medicago sativa*	lucerne
Fabaceae	*Melilotus albus*	white melilot, white sweet clover, honey clover
Fabaceae	*Trifolium repens*	white clover
Onagraceae	*Epilobium tetragonum*	square-stalked willowherb
Onagraceae	*Oenothera biennis*	common evening-primrose, evening star, sun drop, weedy evening primrose
Rubiaceae	*Galium aparine*	cleavers, clivers, bedstraw, goosegrass, catch-weed, stickyweed, stickybud, robin-run-the-hedge, sticky willy, sticky willow, stickyjack, sticklejack, grip gras
Salicaceae	*Populus nigra*	black poplar
Urticaceae	*Urtica dioica*	common nettle, stinging nettle, nettle leaf
MONOCOTYLEDONAE		
Poaceae	*Hordeum vulgare*	barley
Poaceae	*Phragmites australis*	common reed

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Annex D
(informative)

Variation of the Omega-3 index as function of harvest time, plant size and leaf development

In order to assess the robustness of the test, some experiments were carried out to determine (in one area) whether the size of individuals, the stage of development (age) of the harvested leaf, and/or the harvest time influence the results or not. We analysed in this area: four species (*Dittrichia viscosa*, *Blackstonia perfoliata*, *Onobrychis viciifolia* and *Lotus corniculatus*) and six individuals per species.

Experiments as a function of harvest time (9 am, 2 pm, 7 pm), were carried out with mature leaves from medium-sized individuals, experiments as a function of plant size (small (S), medium (M), high (H)) were carried out at 11 am with mature leaves, and experiments as a function of the stage of development (age) of the harvested leaf (young leaves (Y), developing/mature leaves (DM), mature leaves (M) and oldest leaves (O)) were carried out at 11 am with medium-sized individuals (see Figure D.1, Figure D.2 and Figure D.3).

Looking at results of all species included as a function of harvest time (and obtained by calculating an X_p/X_{max} ratio for each individual of a given species as described in 8.3, all harvest times included), no significant differences (Kruskal-Wallis test: p = 0,612) appeared. Nevertheless, as a precautionary measure, we would recommend to not harvest one area in the morning, and another one in the afternoon. This is really feasible because only a few minutes are needed to harvest one species in an area.

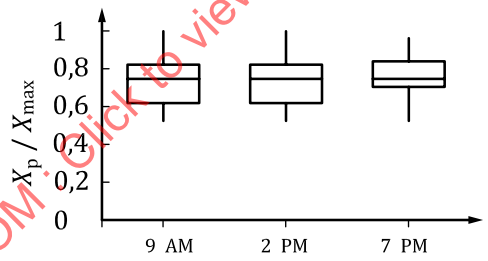

Figure D.1 — Variation in the Omega-3 index as a function of harvest time

Results for the effect of plant size (all species included) were obtained as described in 8.3, by calculating an X_p/X_{max} ratio for each individual of a given species, all sizes included. They showed significant differences (Kruskal-Wallis test: p = 0,007). A similar result was previously observed with *Populus nigra* grown both in a control and in a contaminated area[12]. Hence, special care should be taken for the size of individuals, and it is recommended to harvest leaves on individuals 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.

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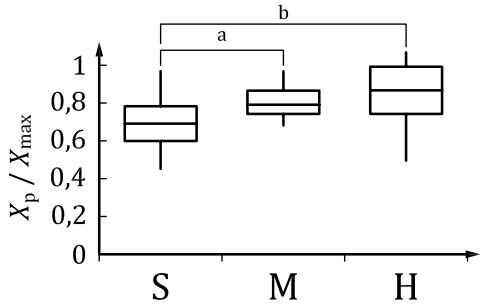
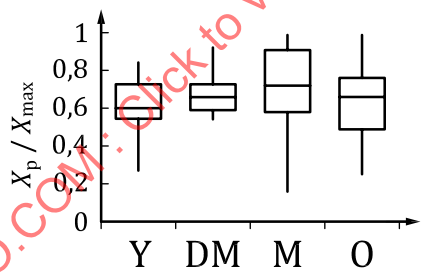


Key

S small
M medium
H high
Dunn Test:
a $p < 0,05$
b $p < 0,002$

Figure D.2 — Variation in the Omega-3 index as a function of plant size

Results for all species included as a function of the stage of development (age) of the harvested leaves (young leaves (Y), developing/mature leaves (DM), mature leaves (M) and oldest leaves (O)) showed no significant differences (Kruskal-Wallis test: $p = 0,274$). Nevertheless, the youngest leaves were not analysed in the present study, and it was previously shown that the Omega-3 index in *Lactuca sativa* leaves varied as a function of age, at least when young seedlings were sampled[6,14]. Hence, as a precautionary measure, we recommend harvesting mature leaves and to disregard developing ones.



Key

Y young leaf
DM developing/mature leaf
M mature leaf
O oldest leaf

Figure D.3 — Variation in the Omega-3 index as a function of leaf development

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