INTERNATIONAL STANDARD

**ISO 23753-2**

Second edition
2019-02

# Soil quality — Determination of dehydrogenases activity in soils —

## Part 2: Method using iodotetrazolium chloride (INT)

*Qualité du sol — Détermination de l'activité des déshydrogénases dans les sols —*

*Partie 2: Méthode au chlorure de iodotétrazolium (INT)*



Reference number
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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).

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

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For an explanation of 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 www.iso.org/iso/foreword.html.

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

This second edition cancels and replaces the first edition (ISO 23753-2:2005), which has been technically revised. The main changes compared to the previous edition are as follows:

— a new Clause 5 “Limitations” has been added;

— in Clause 6, reagents and their preparation have been updated to new results (e.g. concentration of Tris buffer of 100 mmol/l at pH 7,6, incubation time between 4 h to 6 h);

— new Tables 1 and 2 have been added;

— Clause 10 "Validity criteria" has been added;

— a new Annex A “Results of modified parameters” has been added;

— references in Clause 2 and the Bibliography have been updated.

A list of all the parts in the ISO 23753 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

The soil microflora is responsible for the decomposition and conversion of organic substances, carbon, nitrogen, sulfur and phosphorus cycles, soil aggregates stability and as a food source for microbivores. Dehydrogenases, as intracellular enzymes and respiratory chain components of the microbial cells, play a major role in the production of energy by organisms. They oxidize organic compounds by transferring electrons to an acceptor (e.g. $NAD^+$). Dehydrogenases are essential components of the enzyme system of microorganisms. Dehydrogenase activity can therefore be used as an indicator of biological redox systems and as a measure of the viable and physiologically active soil microbial community.

Microbial oxidative activity in soil is linked to respiratory activity, which could be approached with the determination of dehydrogenases activity. Basal and induced respiration in soil could be affected by soil management, practices and contamination.

# Soil quality — Determination of dehydrogenases activity in soils —

# Part 2:
# Method using iodotetrazolium chloride (INT)



## 1 Scope

This document specifies a method for determining activity of dehydrogenases in soil, using 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT)[1]-[5]. As the INT reduction is less sensitive to $O_2$, the method is more robust than the TTC-method described in ISO 23753-1.

## 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 11465, *Soil quality — Determination of dry matter and water content on a mass basis — Gravimetric method*

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 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/

## 4 Principle

INT solution is added to a soil sample and the mixture is incubated at 25 °C ± 1 °C for 4 h to 6 h depending of soil uses (agricultural or forest soil for exemple). The iodonitrotetrazolium formazan (INTF) released is extracted with acetone and quantified by spectrophotometry at a wavelength of 485 nm.

NOTE 1 The method is based on a modified version of the method reported in Reference [1].

NOTE 2 Acetone is used as extractant and samples are not extracted to completion.

## 5 Limitations

— The storage can affect the enzyme activity and hence dehydrogenases activity of samples with different storage times should not be compared.

— For upper layers (L, F, H horizons) of forest humus forms[6] or soils showing high organic matter[7], this method gives very low and variable values.

# 6 Reagents and materials

## 6.1 Soil

Soil samples shall be taken and prepared as specified in ISO 18400-206. If samples which have been sieved in the fresh state are not analysed immediately, they may be kept for up to seven days at 4 °C ± 2 °C. Determine the dry matter content of the sample in accordance with ISO 11465.

## 6.2 **Hydrochloric acid (HCl)**, *c* = 1 mol/l.

## 6.3 **Tris buffer solution,** *c* = 100 mmol/l, pH 7,6.

— Tris(hydroxymethyl)aminomethane (CAS N°: 77-86-1 – 121,14 g/mol): 12,12 g;

— deionized water, *ad*: 1 000 ml;

— hydrochloric acid (CAS N°7647-01-0) (1 mol/l).

Dissolve 12,12 g of Tris base into 800 ml deionized water and adjust pH to 7,6 with hydrochloric acid (1 mol/l). Fill in to 1 000 ml. The storage duration shall not exceed one month at 4 °C ± 2 °C.

## 6.4 **Substrate solution (INT)**, *c* = 15 mmol/l.

— 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride (INT): (CAS Number: 146-68-9 – 505,7 g/mol): 380 mg;

— Tris buffer 100 mmol/l, pH 7,6.

Into a 100 ml conical flask, dissolve 0,38 g of INT into 50 ml Tris buffer 100 mmol/l, pH 7,6, and homogenized it on a magnetic stirrer. Treat the mixture with an ultrasonic probe for 1 min to 2 min, so that the INT is dissolved as much as possible and a suspension is obtained. The storage duration shall not exceed one week at 4 °C ± 2 °C, in the dark.

## 6.5 **Analytical grade acetone.**

## 6.6 **INTF stock solution.**

— Iodonitrotetrazolium formazan (INTF), *c* = 1 µmol/ml (CAS Number: 7781-49-9 – 471,25 g/mol): 47 mg;

— acetone.

Weigh 47 mg of iodonitrotetrazolium formazan into a 100 ml-volumetric-flask, dissolve it in acetone and make up to 100 ml.

# 7 Apparatus

## 7.1 **Visible light spectrophotometer.**

## 7.2 **pH-meter.**

## 7.3 **Suitable glass U-bottom tubes (35 ml to 50 ml), volumetric flasks, pipettes and glass cuvettes**.

For example 2 cm diameter and at least 30 ml to 50 ml capacity (test portion 5 g, see also the Note in Clause 8).

## 7.4 **Incubator**, capable of being set to 25 °C ± 1 °C.

**7.5 Centrifuge**, with temperature regulation at 20 °C ± 1 °C and acceleration of 2 000*g*.

**7.6 Ultrasonic probe.**

**7.7 Orbital tube shaker.**

## 8 Procedure

### 8.1 Establishment of standard curve

Standard curve require several concentrations of iodonitrotetrazolium formazan (INTF), at least in duplicates, preferably in triplicates; all the volumes are giving per tubes.

The stock solution of INTF with a concentration of 1 µmol/ml is used to produce the calibration curve. Distribute the volumes needed into tubes for concentrations 0 nmol/ml, 10 nmol/ml, 20 nmol/ml, 40 nmol/ml, 60 nmol/ml, 80 nmol/ml, in replicate (Table 1).

**Table 1 — Preparation of standard curve of iodonitrotetrazolium formazan in tubes**

| [INTF] nmol/ml | 0 | 10 | 20 | 40 | 60 | 80 |
|---|---|---|---|---|---|---|
| INTF (ml) | 0 | 0,05 | 0,10 | 0,20 | 0,30 | 0,40 |
| Tris buffer pH 7,6 (ml) | 1 | 1 | 1 | 1 | 1 | 1 |
| Acetone (ml) | 4 | 3,95 | 3,90 | 3,80 | 3,70 | 3,60 |

Since INT and INTF are light-sensitive, the solutions should be protected from exposure to light throughout the analysis.

### 8.2 Sampling

The sieved soil is homogenized and four tubes are prepared between 2,0 g ± 0,1 g (reduced sample) to 5,0 g ± 0,1 g (large sample). Table 2 summarizes the protocol.

Briefly, substrate solution (6.4) is added to three replicates of samples and Tris buffer solution (6.3) was added in the control tube instead of the substrate solution (6.4). Homogenize the samples on the test tube shaker, seal the tubes with rubber stoppers and incubate them at (25 °C ± 1 °C in the dark for 4 h to 6 h).

In order to extract the formazan formed, add acetone to the samples (Table 2) and allow them to stand for 1 h in the dark, under orbital agitation (250 $min^{-1}$). Tubes are then centrifuged 5 min at 2 000*g*, 20 °C and the supernatants of each tubes transferred to a glass cuvette. Absorbance shall be read at 485 nm within one hour, on a spectrophotometer after zeroing the apparatus with acetone. The concentration of INTF produced is then calculated using linear model of standard curve.

**Table 2 — Specific details for dehydrogenase activity measurement**

| | Reduced sample | | Large sample | |
|---|---|---|---|---|
| | | control | | control |
| **Soil** | 2 g (in triplicates) | 2 g | 5 g (in triplicates) | 5 g |
| **Substrate solution** | 2 ml | | 5 ml | |
| **Tris 100 mmol/l, pH 7,6** | | 2 ml | | 5 ml |
| **Incubation time** | 4 h to 6 h | | | |
| **Acetone** | 8 ml | 8 ml | 20 ml | 20 ml |
| **Incubation time** | 1 h under orbital agitation (250 $min^{-1}$) | | | |
| **Centrifugation** | 5 min, 2 000*g*, 20 °C | | | |
| **Reading** | Supernatant in glass cuvette at $\lambda$ = 485 nm | | | |

NOTE The modifications to the protocol parameters, in comparison with the previous edition (ISO 23753-2:2005), are justified in Annex A.

## 9 Calculation

The standard curve is plotted for INTF concentration (nmol/ml) versus absorbance. The concentration of INTF produced are determined with the linear model of the standard curve (nmole/ml) and then the dehydrogenases activity (A) calculated using Formula (1):

$$A = \frac{(C_s - C_b) \times V}{m \times DM \times RT} \quad (1)$$

where

$A$ is the enzymatic activity in mU/g of each dry sample replicate (nmol/min/g of dry sample);

$C_s$ is the concentration of INTF formed in sample replicates in nmol/ml;

$C_b$ is the concentration of INTF formed in control tube in nmol/ml;

$V$ is the reaction volume [ = volume of substrate or buffer solution (2 ml or 5 ml) + volume of acetone (8 ml or 20 ml)], in ml;

$RT$ is the reaction time (min);

$m$ is the soil mass per tube, in g

$DM$ is the sample dry matter content in accordance with ISO 11465, as percentage.

## 10 Validity criteria

### 10.1 Standard curve

The standard curve is valid if the following criteria are met.

— R2 ≥ 0,990;

— the maximum of absorbance for the standard (INTF) 80 nmol/ml shall be between 2,3 to 2,6.

### 10.2 Samples

For samples, the validity criteria are as follows.

— Triplicates coefficient of variation (CV) shall be ≤15 %.

— Differences of absorbance between sample triplicates and control shall be ≥ [0,02 to 0,05] according to spectrophotometer sensitivity and repeatability.

— The colour of the sample solution after acetone incubation shall be between pink and red.

## 11 Test report

The test report shall include the following information:

a) a reference to this document, i.e. ISO 23753-2:2019;

b) the identification of the samples; place, type of soil use, month of collection, etc.;

c) the details on the storage temperature and duration of the samples (6.1);

d) the date of the test;

e) the test results;

f) any details that are optional or deviations from the specifications of this document, and any effects which may have affected the results.

# Annex A
(informative)

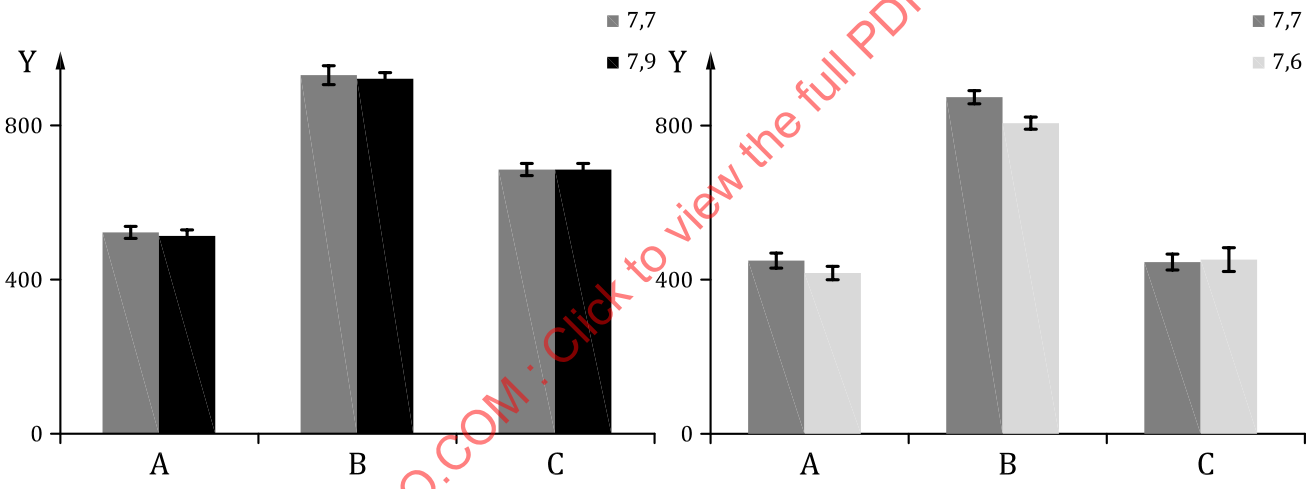
# Results of modified parameters

This document aims to amend and simplify the experimental protocol. Experiments were performed to reduce the amount of soil, to use a same incubation buffer, an optimal incubation time, and a less toxic solvent. Only modifications of incubation buffer, substrate concentration, incubation time to get similar results to those obtained with ISO 23753-2:2005.

The results which led to the modification of the method are described below:

— Effect of pH of incubation buffer

In order to assess the pH buffer effect, Tris 100 mmol/l at pH 7,6 (new proposal) (7,4 and 7,8 in ISO 23753-2:2005) was used. Three different soils were selected to cover standard procedure. Dehydrogenase activities are no different between each test buffer (see Figure A.1), so Tris buffer 100 mmol/l pH 7,6 was chosen for the standard.




**Key**

A alkaline soil (pH = 8,61)
B acidic soil (pH = 5,61)
C neutral soil (pH = 7,1)
Y dehydrogenase activity (mU/g dry soil)

**Figure A.1 — Effect of pH buffer on dehydrogenases activity**

— Effect of incubation time

To determine the optimal incubation time for low and high activities forest soil and loamy clay soil were used. The kinetics of INTF formation and associated activity were followed from 1 h to 24 h and plotted in Figure A.2. There is a linear formation of INTF for 12 h and then a saturation of the system with a non-linear response (green line). According to this curve, dehydrogenase activities are optimal between 4 h to 6 h and then decrease. These incubation times were chosen for this document.