

INTERNATIONAL
STANDARD

ISO
20136

IULTCS/IUC 37

Second edition
2020-06

Leather — Determination of degradability by micro-organisms

Cuir — Détermination de la dégradabilité par les micro-organismes

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Reference numbers
ISO 20136:2020(E)
IULTCS/IUC 37:2020(E)

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Published in Switzerland

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

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 the Chemical Tests Commission of the International Union of Leather Technologists and Chemists Societies (IUC Commission, IULTCS) in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 289, *Leather*, the secretariat of which is held by UNI, in accordance with the agreement on technical cooperation between ISO and CEN (Vienna Agreement).

IULTCS, originally formed in 1897, is a world-wide organization of professional leather societies to further the advancement of leather science and technology. IULTCS has three Commissions, which are responsible for establishing international methods for the sampling and testing of leather. ISO recognizes IULTCS as an international standardizing body for the preparation of test methods for leather.

This second edition cancels and replaces the first edition (ISO 20136:2017), which has been technically revised. The main changes to the previous edition are as follows:

- Method B in the first edition described a closed O₂ circuit system. This system had the inconvenience that, over time, the O₂ concentration decreased and, therefore, so did the activity of the microorganism. Now an open O₂ circuit system has been developed where there is no O₂ limitation and, therefore, the activity of the microorganism is always optimal.
- An explanation about the results calculation method has been added to method B. The CO₂ accumulated in the test (area under the CO₂ moles curve vs time) is calculated.
- The possibility of using municipal wastewater instead of tannery wastewater as an inoculum has been included.
- A new [Annex C](#) has been added which compares the biodegradability with different inoculum sources.

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

One of the main issues faced by the footwear industry is waste treatment. Such wastes, and especially leather, even though they are considered non-hazardous by the regulations in force, are generated in vast quantities and mostly end up in landfills, where natural degradation time is much longer than the product's useful life.

Faced with this problem, there is a growing search for alternative tanning agents that confer the same properties on leather as those provided by the agents currently employed, but which in turn reduce the time to biodegrade in nature.

This document allows the measurement of leather biodegradability in a liquid system by using aerobic microorganisms as an inoculum. The test is considered valid when collagen (positive control) degrades by at least 70 % in a maximum period of 50 days. In order to determine how biodegradable a leather sample (test material) is, its percentage degradability value is compared with the percentage degradability value obtained in collagen, in the same test and period of time. The closer the percentage degradability values, the shorter the time to biodegrade in nature. Therefore, those test materials showing percentage degradability values well below the collagen value will require a longer time for biodegradation in nature.

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Leather — Determination of degradability by micro-organisms

1 Scope

This document specifies a test method to determine the degree and rate of aerobic biodegradation of hides and skins of different animal origin, whether they are tanned or not, through the indirect determination of CO₂ produced by the degradation of collagen.

The test material is exposed to an inoculum (activated sludge from tannery wastewater) in an aqueous medium. If there is not a tannery nearby then urban wastewater can be used as the inoculum.

The conditions established in this document correspond to optimum laboratory conditions to achieve the maximum level of biodegradation. However, they might not necessarily correspond to the optimum conditions or maximum level of biodegradation in the natural medium.

In general, the experimental procedure covers the determination of the degradation degree and rate of the material under controlled conditions, which allows the analysis of the evolved carbon dioxide produced throughout the test. For this purpose, the testing equipment complies with strict requirements with regard to flow, temperature and agitation control.

This method applies to the following materials:

- natural polymers of animal stroma (animal tissue/skins);
- animal hides and skins tanned (leather) using organic or inorganic tanning agents;
- leathers that, under testing conditions, do not inhibit the activity of microorganisms present in the inoculum.

2 Normative references

There are no normative references in this document.

3 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

filter pore No. 1

diffuser with pore size from 100 µm to 160 µm

Note 1 to entry: This measurement is standard.

3.2

inoculum

activated sludge from tannery wastewater

Note 1 to entry: If there is not a tannery nearby then urban wastewater can be used as the inoculum.

4 Symbols and abbreviated terms

atm	the standard atmosphere, a unit of pressure defined as 101 325 Pa
[Ba(OH) ₂]	barium hydroxide
C	carbon
CO ₂	carbon dioxide
GL18	threads are used with H-SA V40/45 Erlenmeyer flasks (5 000 ml volume)
GL14	threads are used with H-SA V29/32 Erlenmeyer flasks (2 000 ml volume)
H-SA V 29/32	inner and outer measures in millimetres of the orifice of the mouth of the Erlenmeyer flasks
H-SA V H40/45	inner and outer measures in millimetres of the orifice of the mouth of the Erlenmeyer flasks
IR	infrared
ppm	parts-per-million (10 ⁻⁶), e.g. 1 mg per kilogram (mg/kg)
PSA	pressure swing adsorption
Q _{nar}	the air flow, in mol, passing through the system per hour (mol/h)
Q _{nCO2}	the CO ₂ air flow, in mol, passing through the system per hour (mol/h)

5 Principle

5.1 General

The procedure consists of the quantification of the CO₂ evolved during the degradation of the polymerised amino acids making up the collagen polymer by the action of microorganisms present in the sludge of tannery biological tanks. The CO₂ evolved is stoichiometrically proportional to the amount of carbon (C) present in said polymer. The initial carbon percentage present in each of the tested samples is determined by elemental analysis. The CO₂ accumulated during the test is converted into biodegradation percentage by means of mathematical equations. The tests shall be conducted in duplicate in the presence of a positive control, comprising minimum test medium (6.3), inoculum (activated sludge from tannery wastewater) and collagen, and a negative control, comprising minimum test medium and inoculum only. The test shall be regarded as valid if the degree of biodegradation of the positive control (pure collagen) is equal to or higher than 70 %.

The tests shall be carried out using equipment able to provide the conditions needed to carry out the test. Agitation, temperature and CO₂-free air inflow should be controlled.

The initial carbon (C) percentage present in the collagen under study is determined by the elemental analysis of the test specimen. The biodegradation percentage does not include the amount of carbon transformed into new cellular biomass that has not been metabolised to carbon dioxide throughout the test.

5.2 Assessment of biodegradation by manual titration; method A

This test method determines the biodegradation percentage of tanned or untanned hides and skins through the indirect measurement of CO₂ evolved during the degradation of collagen, which is the major constituent of the skin, by the action of the microorganisms present in tannery wastewater.

The CO₂ evolved during the test is indirectly determined through the reaction of [Ba(OH)₂] with CO₂, which is precipitated as barium carbonate (BaCO₃). The amount of CO₂ evolved is determined by titrating the remaining non-precipitated [Ba(OH)₂] with a 0,05 mol/l hydrochloric acid solution. These measurements are taken on a daily basis throughout the test.

Biodegradability is assessed by indirectly measuring the CO₂ evolved as a function of time and calculating the biodegradation degree by the difference between the initial carbon percentage present in collagen and the remaining soluble organic carbon content that has not been transformed into CO₂ in the course of the process (see [Figures A.1](#) to [A.3, Annex A](#)).

5.3 Assessment of biodegradation by infrared (IR) detection; method B

With this method, biodegradation is determined through the quantification of the CO₂ evolved throughout the degradation of collagen by means of the direct IR detection and continuous monitoring of the CO₂ concentration using equipment capable of evaluating 12 Erlenmeyer flasks simultaneously (see [Figure B.1](#) to [B.5, Annex B](#)).

The equipment (see [Figure B.1, Annex B](#)) is ready to measure the CO₂ value of several samples contained in different Erlenmeyer flasks. CO₂ evolved during the degradation of the sample by the action of microorganisms is measured by an IR detector. Said detector is managed by a multiplexer system comprising a rotating drum with 12 inlet channels in such a way that every air outlet of the Erlenmeyer flasks is connected to an air inlet of the multiplexer system. The drum is provided with an outlet directly connected to an air flow meter measuring the air flow (l/h) and subsequently to an airtight tank where the CO₂ sensor is located. [Annex B](#) (see [Table B.1](#)) summarizes the parameters, units of measure and range of detection values. Air flow and CO₂ concentration values are saved in a data-capturing system connected to a computer.

6 Chemicals

6.1 Deionised or ultrapure (Milli Q[®]1) water, free from toxic materials with resistivity > 18 MΩ/cm.

6.2 Stock solutions, use only analytical grade reagents. The stock solutions employed in the tests are the same for the two methods used in this document. Prepare synthetic stock solutions by dissolving each of the following in distilled water ([6.1](#)) and made up to 1 l in separate flasks.

6.2.1 Ferric chloride (FeCl₃·6H₂O), 1,00 g.

6.2.2 Magnesium sulfate (MgSO₄·7H₂O), 22,50 g.

6.2.3 Calcium chloride (CaCl₂·2H₂O), 36,43 g.

6.2.4 Phosphate buffer:

- Potassium dihydrogen phosphate (KH₂PO₄), 8,50 g;
- Potassium phosphate dibasic trihydrate (K₂HPO₄·3H₂O), 28,50 g;
- Sodium hydrogen phosphate (Na₂HPO₄), 17,68 g;
- Ammonium chloride (NH₄Cl), 1,70 g.

6.2.5 Ammonium sulfate [(NH₄)₂SO₄], 40,00 g.

1) Milli Q[®] 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 Minimum test medium

The minimum test medium shall contain the following stock solutions diluted to 1 l with deionised water:

6.3.1 Ferric chloride stock solution (6.2.1), 2 ml.

6.3.2 Magnesium sulfate stock solution (6.2.2), 2 ml.

6.3.3 Calcium chloride stock solution (6.2.3), 2 ml.

6.3.4 Phosphate buffer stock solution (6.2.4), 4 ml.

6.3.5 Ammonium sulfate stock solution (6.2.5), 2 ml.

6.4 **Test specimens:** use collagen type I (Sigma^{®2}) or similar) as a positive control. Test specimens shall be basically natural polymers or leather from the tanning industry used for the production of leather clothing.

6.5 **Only for method A:** a [Ba(OH)₂] solution, 0,025 mol/l, is prepared by dissolving 4,0 g [Ba(OH)₂] per litre of distilled water. Filter free of solid material, confirm molarity by titration with standard acid and store sealed as a clear solution to prevent absorption of CO₂ from the air. It is recommended that 5 l be prepared at a time when running a series of tests.

6.6 **Hydrochloric acid**, 0,05 mol/l.

7 Apparatus and materials

The usual laboratory equipment and, in particular, the following:

7.1 **Analytical balance**, capable of reading to 0,000 1 g.

7.2 **Pipettes**, 5 ml to 25 ml capacity.

7.3 **Micro-pipettes**, 500 µl and 1 000 µl.

7.4 **Pre-treatment flasks and flasks** (only for Method A), various sizes.

7.5 **Burettes**, 100 ml.

7.6 **Autonomous CO₂-free air source**, consisting of a noiseless compressor connected to a pressure swing adsorption (PSA) system provided with a molecular sieve.

7.7 **Sepiolite** to filter impurities and humidity from the ventilation system.

7.8 **Stoppers**, flexible non-permeable to CO₂ plastic tubing.

7.9 **Test vessels**

7.9.1 **Method A:** eight 5 l Erlenmeyer flasks (reaction flasks) for each test (two controls and two test specimens per test). 5 000 ml H-SA V H40/50 Erlenmeyer flasks shall be used, as well as V2 distilling heads

2) Sigma[®] 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.

with GL18 threads and filter pore No. 1 diffuser. The volume of the liquid (culture medium + inoculum) shall be 2,5 l in total.

7.9.2 Method B: 12 flasks with a test volume of 1 l (reaction flasks) incorporating a distilling head and an air diffuser which are used to conduct the tests (two controls and four samples in duplicate). The Erlenmeyer flasks shall have a capacity of 2 000 ml with three notches and be of the H-SA V 29/32 (SQ13) model type. They shall incorporate V2 distilling heads with GL14 threads (6 mm air intake and 8 mm air outlet) and filter pore No. 1 diffuser. The volume of the liquid (culture medium + inoculum) shall be 1 l in total.

7.10 Test equipment

7.10.1 Assessment of biodegradation by manual titration (equipment A)

Equipment A operates in such a way that the CO₂-free air is bubbled through a series of seven Erlenmeyer flasks (pre-treatment flasks) that trap residual carbon dioxide in the air flow coming from the PSA device (7.6). The system is then divided into eight lines controlled by eight valves that allow the flow to be independently controlled, which in turn supply eight Erlenmeyer flasks (reaction flasks) located inside the tank. The outlet of each one of the eight Erlenmeyer flasks is directly connected to a series of three glass Erlenmeyer flasks (analysis bottles) connected, each one containing 100 ml of [Ba(OH)₂] 0,025 mol/l, from which the results will be obtained (see Figures A.2 and A.3, Annex A).

The equipment also features a thermostat that allows the regulation of the temperature of the reaction flasks through the recirculation of water in a closed circuit. The test is carried out at 23 °C ± 1 °C. The reaction flasks are constantly agitated at 24 rpm (to-and-fro motion) throughout the entire test duration.

The inoculum volume of each flask varies depending on its degree of activity, ranging between 10 % and 20 % of the total volume (inoculum + minimum test medium), which is 2,5 l. If the inoculum is from urban wastewater the total volume (inoculum + minimum medium) can increase up to 40 % of the total volume.

The air needs to leave the generator through the PSA system which shall have been working for 16 h (overnight) before the start of the test in order to ensure that a stable CO₂ concentration of less than 1 ppm is achieved in the air flow.

During the test, a constant CO₂-free air flow of 150 ml/min is supplied to each reaction flask. The air flow is regularly checked at each outlet by means of scaled flow meters in order to ensure that there are not any leaks in the system.

The quantification of the CO₂ evolved by aerobic digestion of the specimen by microorganisms is carried out by measuring the level of carbonation of 0,025 mol/l [Ba(OH)₂] contained in the three analysis flasks connected to each reaction flask. The analysis flasks are replaced every 24 h with others with the same initial amount of 0,025 mol/l [Ba(OH)₂].

The daily quantification values of the carbonation of [Ba(OH)₂] are entered into a spreadsheet that converts them into biodegradation percentages (Clause 10).

7.10.2 Assessment of biodegradation by IR detection (equipment B)

7.10.2.1 General

The equipment works continuously in an open system in which the air free of CO₂ (7.6) circulates throughout the system impelled by a pump (see Figures B.1 to B.5, Annex B). To increase the amount of oxygen dissolved in the liquid phase, the intake of air into the Erlenmeyer flask is made through the use of an air diffuser incorporated into the distilling head that is in contact with the liquid medium.

The air flow that goes into each Erlenmeyer flask is controlled by a system of individual pressure gauges. The system also features a digital air flow quantification system. Digital data for each

measurement and each Erlenmeyer flask are saved to a file and are subsequently converted into l/h based on a calibration curve.

The equipment is provided with a thermostatic system capable of regulating the temperature of the Erlenmeyer flasks by means of a thermostated tank. Tank water is inside a recirculation system that in turn is connected to a cryothermostat that allows water recirculation at a temperature of $23\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$.

In order to achieve constant agitation of the microbial suspension and samples, the equipment is provided with a system comprising an array of 12 magnets coupled to 12 motors placed underneath the tank base, in such a way that each magnet corresponds to one reaction flask. Agitation of the microbial suspension and samples is achieved by putting a magnet inside each flask. Agitation speed (in revolutions per minute, rpm) is set using specific hardware.

Important quantification parameters are referred to in [Table B.1](#), [Annex B](#).

The CO_2 value of the samples placed in the flasks is sequentially measured by a multiplexer system. This system comprises a rotating drum with 12 inlet channels and one outlet, which is directly connected to an airtight tank where the CO_2 sensor is located. Every air outlet of the reaction flasks is connected to an air inlet of the multiplexer system. By a single rotation, the drum selects just one of the inlets, establishing a direct connection between the selected inlet of one of the reaction flasks and the tank where the detector is located, and blocking the rest of the inlets. A stepper motor makes the multiplexer system rotate to select the relevant inlet, and specifically designed hardware and the corresponding firmware control which of the 12 inlets is selected at all times. This way, it is possible to save the information relative to the CO_2 evolved (ppm) and the respective air flow (l/h) of a given flask at a given time.

The minimum test medium and the inoculum are added to the Erlenmeyer flasks. The volume of the inoculum in each flask varies according to the degree of activity, ranging between 10 % and 20 % of the total volume (inoculum + minimum test medium), which amounts to 1 l. If the inoculum is from urban wastewater the total volume (inoculum + minimum medium) can increase up to 40 % of the total volume.

Then the inlet and outlet connectors of the CO_2 detector are installed. The agitation and the temperature are switched on and the test is started on the computer, keeping it in operation for a period of 16 h (overnight) in order to properly condition the microorganisms present in the medium. Afterwards, collagen (in the positive controls) and leather (in the samples) are added.

The biodegradation equipment features software capable of controlling and recording the values of the CO_2 (ppm) and air flow (l/h) produced in each flask during the test at intervals defined by the user.

7.10.2.2 Equipment B calibration system

Two different types of calibration are performed: CO_2 sensor and air flow.

7.10.2.2.1 CO_2 sensor calibration: the calibration of the CO_2 detection equipment is carried out using special gas mixtures with different CO_2 concentrations (50 ppm, 150 ppm and 300 ppm), in addition to a gas mixture with 99,9 % O_2 as zero CO_2 concentration. At the end of the calibration process, a linear calibration curve between 0 ppm and 2 000 ppm is traced according to [Formula \(1\)](#):

$$y = mx + c \quad (1)$$

where

y and x are the variables;

m is the slope of the line;

c is the point at which the line crosses the y -axis;

and the respective coefficient of determination (R^2).

7.10.2.2.2 Air flow calibration: the digital flow is calibrated independently for each flask using rotameters (one for each flask) positioned before the CO₂-free air inlet of each of the 12 flasks.

All air flow values are stored in a software program specifically developed for this test, which also makes it possible to control the parameters and save all data of CO₂ evolved during the test in the different reaction flasks.

8 Procedure

8.1 Collection and preparation of the inoculum

Use samples (wastewater) collected from a tannery aerated biological tank as inoculum. If there is no tannery nearby, urban wastewater can be used as inoculum, see the comparative biodegradability in [Annex C](#). The sample shall be free from large inert objects, such as leather pieces, which shall be removed manually.

The sample shall be taken immediately to the laboratory in a portable cool box so as to maintain its original characteristics. Decant the sample to remove impurities and, in order to reduce the amount of suspended solids, the wastewater should be filtered with glass wool. Alternatively, centrifuge the sample at 1 500 min⁻¹ for 5 min.

8.2 Preparation of the test material and reference material

All specimens shall be in ground or shredded form and weighed before being added to the Erlenmeyer flasks. The initial concentration of the specimens shall be between 0,18 g/l and 0,19 g/l, the absolute amount for each test being 0,5 g (method A) and 0,18 g to 0,19 g (method B).

The test materials as the collagen type 1 (positive control material) shall be dry before being added to the Erlenmeyer flasks. In the case of wet-blue or wet-white skins, they should first be dried in an oven at 70 °C for a minimum period of 24 hours or until they are dry.

8.3 Test conditions and incubation period

With the exception of test samples, all laboratory materials shall be autoclaved before use. All test solutions and culture media (other than the inoculum) shall also be autoclaved before use, except for ferric chloride, which shall be filtered in sterile conditions.

First, the minimum test medium and the inoculums shall be added to each flask. The agitation and the temperature are switched on, keeping it in operation for a period of 16 h (overnight) in order to properly condition the microorganisms present in the medium. Afterwards, collagen (in the positive controls) and leather (test materials) are added and the test is started.

Check the activity of the inoculum during the test by means of a biodegradable reference material, preferably ground or shredded type 1 collagen and by measuring CO₂ evolution during its degradation. The reference material shall be degraded by 70 % or more at the end of the test in order to be considered valid. Because of the possibility of the inoculum presenting suspended organic compounds, flasks containing inoculum and culture medium shall be used as a negative control. The values for CO₂ evolved in these flasks shall be subtracted from the values evolved in the positive control and the specimens.

8.4 Termination of the test

The test should be considered terminated when the collagen sample (positive control) has attained the plateau phase with biodegradation values equal to or higher than 70 % of initial carbon.

When 70 % degradation of the positive control (pure collagen) is achieved, the test may continue for a few more days if the samples show log phase kinetics of CO₂ production, thus giving enough time for the sample to achieve its maximal biodegradation.

When the positive control shows biodegradation values lower than 70 % after 50 days, the test shall be discarded and repeated.

9 Quantification

9.1 Assessment of biodegradation by manual titration (method A)

9.1.1 Determination of the organic carbon content

The total organic carbon content of the material being tested is determined by elemental analysis. This allows the theoretical maximum quantity of carbon dioxide evolution to be calculated.

The test material has w % (percentage by mass) of carbon as determined by the elemental analysis in [Formula \(2\)](#) and [Formula \(3\)](#):

$$m_{TC} = w/100 \cdot m_s \quad (2)$$

where

m_{TC} is the carbon in test material added to the Erlenmeyer flask (g);

w is the % mass fraction of carbon in test material from elemental analysis (%);

m_s is the mass of test material added to the Erlenmeyer flask (g).



This means 12 g of C would yield 44 g CO_2 . The theoretical maximum quantity of CO_2 is shown by [Formula \(4\)](#):

$$m_{TCO_2} = 44/12 \cdot m_{TC} \quad (4)$$

where m_{TCO_2} is the maximum quantity of CO_2 (g).

9.1.2 Determination of the amount of CO_2 produced

Correct for the amount of carbon dioxide produced with the negative control (culture medium + inoculum) by subtracting negative control titration from test material titration obtained with 0,05 mol/l HCl, using [Formula \(5\)](#) and [Formula \(6\)](#).



This means x moles of CO_2 is equal to $x/2$ moles of HCl.

9.1.3 Correcting for normality of HCl

The amount of CO_2 (mol), x_{CO_2} , is determined according to [Formula \(7\)](#):

$$x_{CO_2} = (c_{HCL} \cdot t_{HCL})/2 \quad (7)$$

where

c_{HCL} is the concentration of HCl (mol/l);

t_{HCL} is the titre volume obtained with 0,05 mol/l HCl (ml).

The amount of CO_2 evolved from test material (mg), m_{CO_2} , is determined according to [Formula \(8\)](#):

$$m_{\text{CO}_2} = [(c_{\text{HCL}} \cdot t_{\text{HCL}} \cdot 44)/2] \quad (8)$$

For a titration with 0,05 mol/l HCl, (i.e. $c_{\text{HCL}} = 0,05$) the amount of CO_2 (mg) is as shown in [Formula \(9\)](#):

$$m_{\text{CO}_2} = 1,1 \cdot t_{\text{HCL}} \quad (9)$$

Hence, the amount of carbon dioxide evolved (mg) is obtained by multiplying the HCl titration by 1,1.

9.1.4 Percentage of biodegradation from CO_2 evolved

The percentage of biodegradation of the test material determined from carbon dioxide evolved is calculated as shown in [Formula \(10\)](#):

$$B_{\text{CO}_2} = [(m_{\text{CO}_2} \cdot 1\,000) / m_{\text{TCO}_2}] \cdot 100 \quad (10)$$

where B_{CO_2} is the percentage biodegradation of test material (%).

9.2 Assessment of biodegradation by IR (method B)

9.2.1 Determination of the organic carbon content

The total organic carbon content of the material being tested is determined through an elemental analysis. This allows the maximum amount of CO_2 that can be generated in each test run to be theoretically calculated.

The test material has w % (percentage by mass) of carbon as determined by the elemental analysis in [Formula \(11\)](#) and [Formula \(12\)](#).

$$m_{\text{TC}} = w/100 \cdot m_s \quad (11)$$

where

m_{TC} is the carbon in test material added to the Erlenmeyer flask (g);

w is the mass fraction of carbon in test material from elemental analysis (%);

m_s is the mass of test material added to the Erlenmeyer flask (g).



This means 12 g of C would yield 44 g CO_2 . The theoretical maximum quantity of CO_2 is as shown in [Formula \(13\)](#):

$$m_{\text{TCO}_2} = 44/12 \cdot m_{\text{TC}} \quad (13)$$

where m_{TCO_2} is the theoretical maximum quantity of CO_2 (g).

9.2.2 Determination of the amount of CO₂ produced

CO₂ produced during the degradation of samples is measured by an IR detector present in the quantification equipment. For this purpose, the sensor is previously calibrated between 0 ppm and 2 000 ppm with commercial mixtures of calibration gases ([7.10.2.2.1](#)).

9.2.3 Percentage of biodegradation from CO₂ data

9.2.3.1 General

The percentage of biodegradation from carbon dioxide produced is calculated from the conversion of ppm of CO₂ produced during the test, taking into account the average of the values obtained in the two Erlenmeyer flasks of each sample (duplicate tests). After a series of mathematical calculations, these values are converted into percentage of biodegradation. To this end, the following parameters shall be considered:

- air flow ([7.10.2.2.2](#)) going in (O₂, N₂) and gaseous mixture flow going out (O₂, N₂, CO₂) of each reactor (Q_v), expressed in l/h;
- air temperature within the system, expressed in Kelvin (K);
- total pressure (P_{total}) is the total pressure inside the Erlenmeyer flask that comprises the atmospheric pressure, which is dependent on the altitude of the location and the manometric pressure, expressed in atm;
- reactor volume, expressed in litres (l);
- CO₂ fraction on exiting the IR detector (Y_{CO_2}), expressed in parts per million (ppm CO₂).

The air flow (Q_v) expressed in l/h is converted into air flow (Q_{nar}) expressed in mol/h applying [Formula \(14\)](#):

$$(P_{\text{total}} \cdot Q_v = Q_{\text{nar}} \cdot R \cdot T) \quad (14)$$

where

P_{total} is the total pressure inside the Erlenmeyer flask that comprises the atmospheric pressure, which is dependent on the altitude of the location, and the manometric pressure; expressed in atm;

Q_v is the air flow, in litres, passing through the system per hour (l/h);

Q_{nar} is the air flow, in mol, passing through the system per hour (mol/h);

R is the gas constant (0,082 atm · l · mol⁻¹ · K⁻¹);

T is the temperature of the gaseous phase in Kelvin (K), where K = °C + 273,15 °C.

This formula allows the determination of the number of moles of the gas mixture as a reference for the transformation of CO₂ ppm evolved during the test into percentage of biodegradation. For this, it is necessary to transform, for each measure throughout the test, the air flow (Q_{nar}) expressed in mol/h into CO₂ air flow (Q_{nCO_2}) expressed in mol/h, as shown in [Formula \(15\)](#):

$$Q_{\text{nCO}_2} = Q_{\text{nar}} \cdot Y_{\text{CO}_2} \quad (15)$$

where

Q_{nCO_2} is the CO_2 air flow, in mol, passing through the system per hour (mol/h);

Q_{nar} is the air flow, in mol, passing through the system per hour (mol/h);

Y_{CO_2} is the CO_2 fraction in air flow exiting the IR detector (ppm).

The inlet air flow is equal to the outgoing gas flow, since for each mol of O_2 consumed 1 mol of CO_2 is produced.

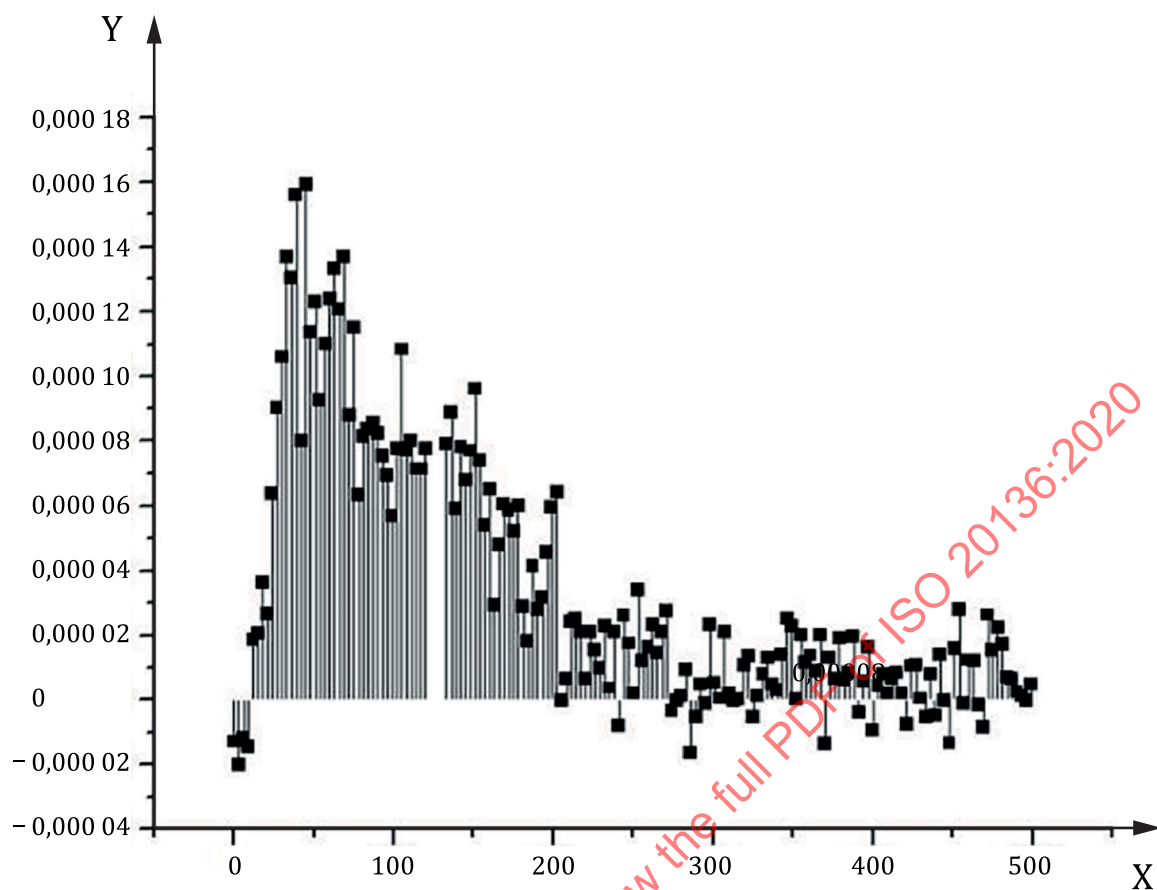
After calculating the average Q_{nCO_2} of each sample, and for each sampling point, the Q_{nCO_2} of the negative control corresponding to each sampling point shall be subtracted from the Q_{nCO_2} of the sample. This way, it is possible to determine the number of CO_2 moles evolved from the sample or collagen (positive control), from which the CO_2 produced by the inoculum (negative control) has already been deducted.

Therefore, for each sampling point, the CO_2 air flow would be calculated using [Formula \(16\)](#):

$$Q_{nCO_2} = Q_{nCO_2 \text{ test material}} - Q_{nCO_2 \text{ negative control}} \quad (16)$$

9.2.3.2 Expression of the total number of moles of CO_2 evolved

From the Q_{nCO_2} data obtained at the different sampling points, plot a Q_{nCO_2} (mol/h) graph as a function of time (h) and then calculate the total number of moles of CO_2 evolved at different time intervals (e.g. every 2 h) until the test is terminated. To this end, the area under the curve, which can be obtained using graphic software, traced by the Q_{nCO_2} graph as a function of time shall be integrated, for which a specific mathematical program shall be used, see [Figure 1](#).



Key

X time (h)

Y Q_{nCO_2} (mol/h)

Figure 1 — Graph of the integration area of a sample (Q_{nCO_2} vs time)

9.2.3.3 Conversion of moles of CO₂ evolved into mg of CO₂ evolved

Calculate the milligrams of CO₂ evolved using [Formula \(17\)](#):

$$m_{\text{CO}_2} = x_{\text{CO}_2} \cdot 44 \quad (17)$$

where

m_{CO_2} is the amount of CO₂ evolved from test material degradation (g);

x_{CO_2} is the amount of CO₂ evolved from test material degradation (mol), from which the mol CO₂ evolved from the inoculum at that very same time have been deducted. The value of the integrated area at a given time is given in [9.2.3.2](#).

9.2.3.4 Calculation of the CO₂ mass present in the initial test sample

From the carbon (C) percentage present in the leather or collagen test material, previously determined by elemental analysis ([9.2.1](#)), it is possible to calculate the total C amount, in mg, present in the initial test material sample and, hence, the maximum CO₂ value that this C mass can produce.

From the initial mass multiplied by the C percentage present in the test material, calculate the theoretical maximum amount of carbon that can be transformed into CO₂ during the test using [Formula \(18\)](#):

$$m_{\text{TC}} = w/100 \cdot m_s \quad (18)$$

where

m_{TC} is the mass of carbon in test material sample (mg);

w is the mass fraction of carbon in test material from elemental analysis (%);

m_s is the initial mass of test material sample (mg).

9.2.3.5 Calculation of the CO₂ mass evolved from the initial sample

From the C theoretical maximum, calculate the CO₂ theoretical maximum than can evolve during the test using [Formula \(19\)](#):

$$m_{\text{TCO}_2} = 44/12 \cdot m_{\text{TC}} \quad (19)$$

where m_{TCO_2} is the theoretical maximum quantity of CO₂ (g).

Calculate CO₂ evolved during a given time interval throughout the test (m_{CO_2}) by multiplying the number of moles of CO₂ produced by the test material degradation, during said time interval, by the molecular mass of CO₂, according to [Formula \(17\)](#) in [9.2.3.3](#).

9.2.3.6 Calculation of the percentage of biodegradation

In order to calculate the percentage of biodegradation at any time, divide the accumulated CO₂ (g) by the theoretical maximum CO₂ that can evolve from the initial C (g) multiplied by the percent factor (100), as shown in [Formula \(20\)](#), see also [Figure 2](#).

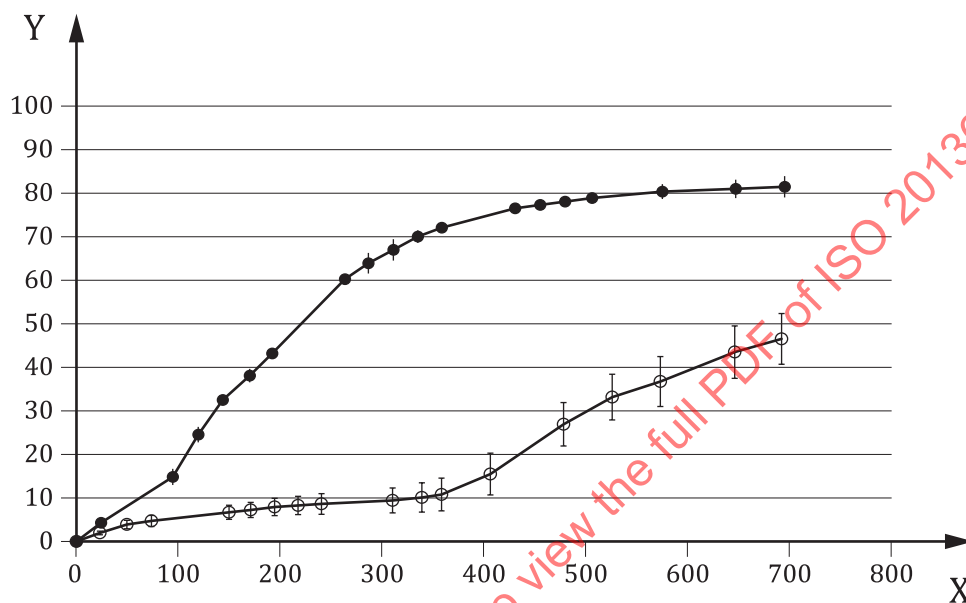
$$B_{\text{CO}_2} = (m_{\text{CO}_2}/m_{\text{TCO}_2}) \cdot 100 \quad (20)$$

where

B_{CO_2} is the percentage biodegradation of test material (%);

m_{CO_2} is the CO_2 evolved during a given time interval throughout the test (g);

m_{TCO_2} is the theoretical maximum quantity of CO_2 that could evolve (g).



Key

X time (h)

Y biodegradation (%)

● collagen

○ test material

Figure 2 — Graph of percentage of biodegradation vs time

9.2.3.7 Calculation sequence for the determination of sample biodegradation

- From the CO_2 (ppm) and air flow (l/h) data obtained throughout the whole test, calculate the average for the two Erlenmeyer flasks of each test material, and of the positive (collagen) and negative (inoculum + minimum test medium) controls.
- Using the calculations of 9.2.3.1, determine the average Q_{nar} values of the test materials and controls for each sampling point during the test.
- For each sampling point, subtract the Q_{nar} of the negative control (9.2.3.1) from the Q_{nar} of the test materials and the positive control (9.2.3.1).
- From the table of values obtained in point c), and the respective sampling times, plot a graph for each test material and positive control, showing the Q_{nar} as a function of time (h) (9.2.3.2).
- From the graph, integrate the graph area under the curve every 24 h of testing (9.2.3.2). The value obtained after the integration of the graph area corresponds to the C mass, in mg, that has been transformed into CO_2 until the time (h) integrated in the graph.

- f) Finally, for each value calculated in point e), calculate the biodegradation percentage of the samples or positive control (collagen) according to [9.2.3.6](#).

10 Expression of results

The percentage of degradation of each test material in comparison with collagen is presented as a relative percentage, calculated on the basis of the absolute value of collagen degradation converted to 100 %, according to [Formula \(21\)](#):

$$B_{\text{rel}} = (B_{\text{CO}_2} \cdot 100) / A \quad (21)$$

where

B_{rel} is the relative biodegradability of each test material (%);

B_{CO_2} is the measured biodegradation of each test material (%);

A is the measured biodegradation of collagen (%) (70 % or more).

11 Validity of results

The test shall be regarded as valid if the degree of biodegradation of the reference material in the reaction flasks is equal to or higher than 70 % in absolute terms (see [Clause 10](#), term A).

12 Test report

The test report shall include at least the following:

- a reference to this document, i.e. ISO 20136:2020;
- information on the inoculum including source, date of collection, storage, handling and potential acclimation to test material;
- carbon (C) content of the test material, both the collagen (positive control) and the natural polymer or leather samples;
- accumulative average carbon dioxide evolution over time until plateau should be reported and displayed graphically as lag-phase and slope (rate);
- % absolute biodegradation (B_{CO_2}) and relative biodegradability (B_{rel}) of each test material;
- the date of the test.

Annex A (informative)

Determination of the degree and rate of degradation of the material

The biodegradability assessment equipment is a compact unit that has been specifically conceived for testing biodegradation of leather. However, its applicability can be extrapolated to the study of the biological degradation of any textile (e.g. fabrics, weaves) or polymeric material (plastics), as long as the relevant adjustments are made to the methodology, especially with regards to the inoculum and positive control used. The test method therefore covers the determination of the degree and rate of degradation of the material under controlled laboratory conditions based on the analysis of the evolution of CO₂ throughout the test. For this purpose, the unit complies with strict requirements referring to flow control (CO₂-free air), thermal control and agitation control (to-and-fro motion). This unit was developed to simplify the experimental process, allowing all the operational controls to be accessed from one easily accessible and understandable control panel situated on the top of the equipment ([Figures A.1](#) and [A.2](#)).



Figure A.1 — View of the unit for biodegradability assessment

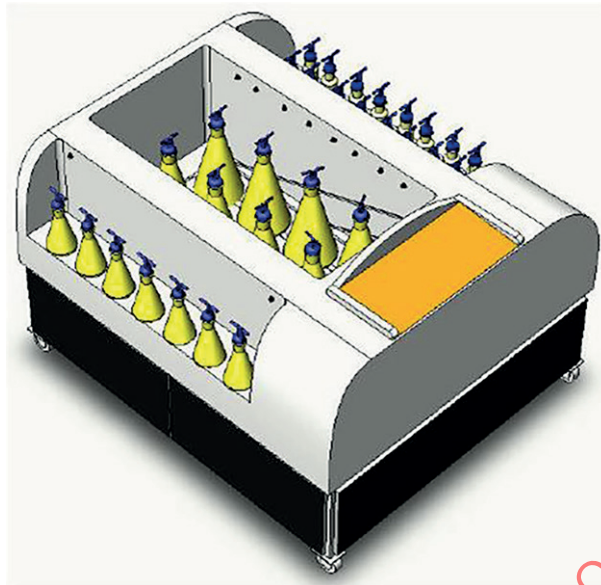
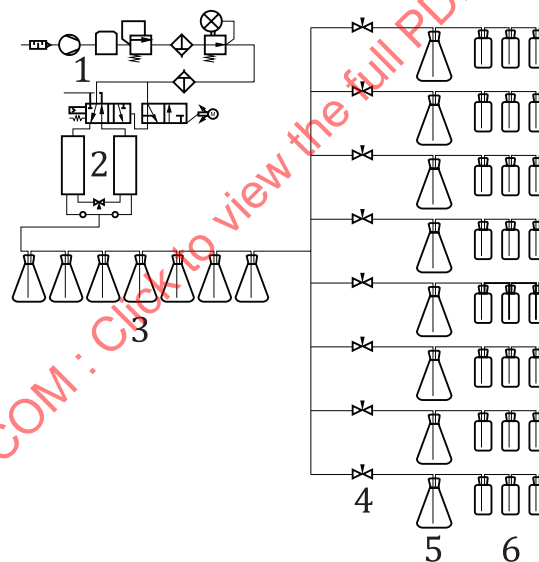


Figure A.2 — Volumetric view of the unit for biodegradability assessment



Key

- 1 air pump
- 2 PSA system
- 3 pre-treatment flasks
- 4 flow meter
- 5 Erlenmeyer flasks
- 6 analysis flasks

Figure A.3 — Diagram of the unit for biodegradability assessment

Figure A.3 shows a diagram of the unit for biodegradability assessment from the point of view of the basic experimental procedure. As can be seen, the unit is provided with an autonomous clean CO₂-free air generation system consisting of a noiseless compressor (specially conceived for a non-industrial use in research laboratories, with a noise level < 40 dB) and a CO₂ filter or trap (PSA system). The generated CO₂-free air is bubbled through a series of seven Erlenmeyer pre-treatment flasks and this is then divided into eight lines where the flow is independently controlled, which in turn supply eight

Erlenmeyer reaction flasks located inside the tank. The outlet of each one of the eight Erlenmeyer flasks is directly connected to a series of three Erlenmeyer analysis flasks, where the CO₂ evolved during the degradation of the specimen is trapped for its subsequent quantification.

The unit also features a thermostat that allows temperature control and regulation inside the tank through the recirculation of approximately 200 l of thermostated water in a closed circuit.

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Annex B (informative)

Quantitative determination of leather biodegradation

The equipment consists of a completely automated system able to precisely and simultaneously measure the percentage of CO₂ evolved during leather degradation by the action of aerobic microorganisms. These data are subsequently converted into biodegradation percentage of leather samples.

The system's capacity is 12 Erlenmeyer flasks and it is managed by a multiplexer system comprising a rotating drum provided with an outlet directly connected to an air flow meter measuring the air flow (l/h) and subsequently to an airtight tank where the CO₂ sensor is located. Air flow and CO₂ concentration values are saved in a data-capturing system connected to a computer.



Figure B.1 — General view of the equipment for CO₂ quantification by IR

The CO₂ IR detection equipment has the following features ([Table B.1](#)):

- thermostated tank with a capacity of 12 flasks of 2,5 l total volume and 1 l test volume;
- multiplexer system comprising a rotating drum with 12 inlet channels in such a way that every air outlet of the reaction flasks is connected to an air inlet of the multiplexer system;
- digital air flow meter (l /h);
- manual system for individual air-flow adjustment in every Erlenmeyer flask made up of a system of pressure gauges;
- PSA system ([7.6](#)) providing CO₂-free air;
- CO₂ detector consisting of a CO₂ sensor able to measure CO₂ concentrations from 0 ppm to 2 000 ppm;
- digital pressure system to quantify the total pressure (ambient pressure + flask reactor pressure);