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Leather — Determination of degradability by micro-organisms

Cuir — Détermination de la dégradabilité par les micro-organismes

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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).

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

This document was prepared by 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 method for the sampling and testing of leather. ISO recognizes IULTCS as an international standardizing body for the preparation of test methods for leather.

Introduction

One of the big problems faced by the footwear industry is waste treatment. Although this waste, especially in the case of leather, is not considered hazardous by current legislation, it is however produced in large quantities which present a problem for municipal landfill sites.

The aim of the tanning process is to avoid skin putrefaction and increase the resistence of the obtained leather. For this purpose, chemical and biological agents are used which are involved in the denaturation and hardening of the main stromal protein, collagen, thus also producing physicochemical changes in the skin.

There is a wide range of different agents used for leather tanning, which can be based on organic products, vegetable extracts or inorganic products, mostly metals.

The most used tanning agent in the footwear industry is Chromium (III), which gives the skin desirable characteristics, such as elasticity, easy buffing and a good breathability and vapour permeability. However, the traditional tanning industry, and especially chrome tanning, generates wastes that pose an environmental threat. Also, chrome-tanned hides and skins have too long a lifespan, much larger than the useful life of the final products. Therefore, the use of additives that are less harmful to the environment and which generate products that have a certain ease of degradation, once the material has achieved its purpose, would be preferred, thus minimising waste products.

Within this sector, the development of fast biodegradability quantification methods for leather that has been treated with alternative tanning agents is needed in order to predict whether these materials are more biodegradable than their predecessors. The methodology described in this document attempts to allow the completion of this form of analysis in a test time of no more than 35 days.

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 CO_2 produced by the degradation of collagen.

The test material is exposed to an inoculum (activated sludge from tannery wastewater) in an aqueous medium.

The conditions established in this document correspond to optimum laboratory conditions to achieve the maximum level of biodegradation. However, they may 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:

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

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

3.1
filter pore no. 1
diffuser with pore size from 100 microns to 160 microns

Note 1 to entry: This measurement is standard.

3.2
inoculum
activated sludge from tannery wastewater

4 Symbols and abbreviated terms

$[Ba(OH)_2]$	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
PSA	pressure swing adsorption

5 Principle

5.1 Method A: assessment of biodegradation by manual titration

This test method determines the biodegradation percentage of tanned or untanned hides and skins through the indirect measurement of CO_2 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 CO_2 evolved during the test is indirectly determined through the reaction of $[Ba(OH)_2]$ with CO_2, which is precipitated as barium carbonate ($BaCO_3$). The amount of CO_2 evolved is determined by titrating the remaining barium hydroxide 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 CO_2 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 CO_2 in the course of the process.

The initial carbon percentage (C) 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 a new cellular biomass that has not been metabolized to carbon dioxide throughout the test.

The tests shall be carried out using equipment able to provide the conditions needed to carry out the test. Agitation, experiment temperature and CO_2-free air inflow should be controlled.

The test shall be carried out in duplicate in the presence of a positive control, which is made up of a synthetic medium, microorganisms and collagen, and a negative control, which is made up only of a synthetic medium and inoculum (activated sludge from tannery wastewater), allowing the assessment of two different leather samples that can be evaluated in duplicate.

5.2 Method B: assessment of biodegradation by infrared detection

With this method, biodegradation is determined through the quantification of the CO_2 evolved throughout the degradation of collagen, by means of the direct IR detection and continuous monitoring of the CO_2 concentration. The equipment comprises a reaction unit made up of a closed set of

unidirectional gas flow recirculation tubes, an aerator immersed in the reaction fluid contained in the reaction flask, a membrane pump for unidirectional flow that makes the gas go through the CO_2 concentration detector area, an IR sensor, and a data capture system connected to a computer.

This system is in its final development stage. The methodology will be added to this document application at a later stage.

The initial percentage of carbon (C) present in the collagen under study is determined through the elemental analysis of each sample. The percentage of biodegradation does not include the quantity of carbon converted into a new cellular biomass which is not metabolised into carbon dioxide during the course of the test.

The tests shall be carried out using equipment able to provide the conditions needed to carry out the test. Agitation, experiment temperature and CO_2-free air inflow should be controlled.

The tests are conducted in duplicate in the presence of a positive control, composed of a synthetic medium, microorganisms and collagen, and a negative control, composed only of a synthetic medium and an inoculum, allowing the assessment of five different leather samples that can be evaluated in duplicate.

6 Chemicals

The reagents employed in the tests are the same for the two methods used in this document (Method A and Method B) only with some adjustments in the volume of the reaction flasks specific of each methodology (method A: a final liquid volume of 2,68 l; method B: a final liquid volume of 1 l).

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

6.2   Test medium: Use only analytical grade reagents.

6.2.1   Prepare synthetic stock solutions by dissolving each of the following in distilled water to 1 l:

6.2.1.1   Ferric chloride ($FeCl_3 \cdot 6H_2O$), 1,00 g.

6.2.1.2   Magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 22,5 g.

6.2.1.3   Calcium chloride ($CaCl_2 \cdot 2H_2O$), 36,43 g.

6.2.1.4   Phosphate buffer KH_2PO_4 8,5 g, $K_2HPO_4 \cdot 3H_2O$ 28,5 g, Na_2HPO_4 17,68 g, and NH_4Cl 1,7 g, for a total of 56,38 g.

6.2.1.5   Ammonium sulfate [$(NH_4)_2SO_4$], 40 g.

6.2.2   The test medium shall contain the following reagents diluted to 1 l with high-quality distilled water:

6.2.2.1   Magnesium sulfate solution, 2 ml.

6.2.2.2   Calcium chloride solution, 2 ml.

6.2.2.3   Phosphate buffer solution, 4 ml.

6.2.2.4   Ferric chloride solution, 2 ml.

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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.

6.2.2.5   Ammonium sulfate solution, 2 ml.

6.3   Test specimens: Use collagen type I (Sigma or similar) as a positive control. Test specimens shall be basically leather from the tanning industry used for the production of leather clothing.

6.4   Only for Method A: Barium hydroxide solution, 0,025 mol/l, is prepared 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 CO_2 from the air. It is recommended that 5 l be prepared at a time when running a series of tests.

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   Volumetric flask, 1 l.

For each method, the following materials should be employed:

7.5   Method A: Assessment of biodegradation by manual titration

7.5.1   Biodegradation test equipment

The procedure is partially automated thanks to the equipment specially conceived for this test (see Annex A and Figure A.1).

This equipment allows four test specimens to be analyzed in duplicate (two test specimens and two controls). It also allows agitation, experiment temperature and CO_2-free air inflow to be controlled.

7.5.2   Autonomous CO_2-free air source, consisting of a noiseless compressor connected to a PSA (pressure swing adsorption) system provided with a molecular sieve, from Peak Scientific, model PG14L.

7.5.3   Sepiolite to filter impurities and humidity from the ventilation system.

7.5.4   Test flasks

7.5.4.1   Eight 5 l Erlenmeyer®[2)] flasks (reaction flasks) for each test (two controls and two test specimens per test). 5 000 ml H-SA V H40/45 Erlenmeyer® flasks shall be used, as well as V2 distilling heads with GL18 threads and a filter pore no. 1 diffuser.

7.5.4.2   Connect the PSA equipment (7.5.2) to four glass flasks and two plastic flasks connected in series using silicone tubing. The first three flasks shall contain 700 ml of 10 mol/l sodium hydroxide (NaOH). The fourth flask shall be empty. The fifth flask shall contain 700 ml of 0,025 mol/l [Ba(OH)$_2$]. The sixth flask shall be empty.

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2)   Erlenmeyer® 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.

7.5.4.3   For each of the Erlenmeyer® flasks in 7.5.4.1, three 0,25 l bottles connected in series using silicone tubing, each one containing 100 ml of 0,025 normal (mol/l) barium hydroxide [Ba(OH)$_2$] to trap CO_2 (analysis flasks).

7.5.5   **Stoppers, flexible non-permeable to CO_2 plastic tubing, 100 ml burettes**.

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

7.6   **Method B: Assessment of biodegradation by IR detection**

7.6.1   **Biodegradation equipment**

The procedure is totally automated through equipment developed specially for these tests (see Annex B and Figure B.1).

This equipment allows the analysis of up to seven samples, in duplicate, per test run (five samples and two controls). The agitation is orbital and the temperature is controlled by an air cooling system. The quantification of CO_2, produced during the leather biodegradation process, is carried out using CO_2 detection equipment that incorporate infrared sensors with a measuring range between 0 % and 5 %.

7.6.2   The supply of air (O$_2$) free of carbon dioxide (CO_2) is provided by a gas mixture of O$_2$:N$_2$ at a ratio of 30:70 injected directly into the reaction flasks for 30 min at a rate of 3 l/min.

7.6.3   **Easy-to-use software** for the capture, processing, and monitoring of signals, with the capacity to store data for long periods of time.

7.6.4   The calibration of the CO_2 detection equipment is carried out with special mixes of gases at different concentrations of CO_2 (1 %, 3 %, and 5 %) in addition to a gas mixture with 99,9 % O$_2$ (oxygen 5,0) with zero CO_2 concentration. At the end of the calibration, a linear calibration curve between 0 % and 5 % is generated according to the linear equation of $Y = AX + B$ and its respective coefficient of determination (R^2).

7.6.5   Calibration values are stored in a software program specially developed for these tests, which additionally allows the test parameters to be controlled and all production data of CO_2 generated during the test in the different reaction flasks to be saved.

7.6.6   **Test vessels**

7.6.6.1   14 flasks with a useful volume of 2 l (reaction flasks) incorporating a distilling head and an air diffuser which are used to conduct the tests (two controls and five 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 a filter pore no. 1 diffuser. The volume of the liquid (culture medium + inoculum) shall be 1 l in total.

7.6.6.2   The flasks shall be connected to the CO_2 detection equipment through nylon tubes. The tube that connects the outlet of the CO_2 detection equipment to the distilling head inlet flange up to the diffuser shall have a diameter of 6 mm. The tube that connects the air outlet of the flask to the inlet of the CO_2 detection equipment should have a diameter of 8 mm.

7.6.7   **CO_2 detection and data capture system**

For each one of the test flasks (7.6.6.1), CO_2 detection equipment is needed. This CO_2 detection equipment is connected to a capture and signal processing unit that manages the signals of each piece of equipment and sends them to a computer data monitoring and storage system. This way, the values of all CO_2 produced during the test in the reaction flasks are saved and remain available on a computer.

8 Procedure

8.1 Collection and preparation of the inoculum

Use samples (wastewater) collected from a tannery aerated biological tank as inoculum. 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 shall 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

Check the activity of the inoculum during the test by means of a biodegradable reference material, preferably powdered type 1 collagen (Sigma or similar) and by measuring CO_2 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 CO_2 evolved in these flasks shall be subtracted from the values evolved in the positive control and the specimens.

For the biodegradation tests, tanned hides and skins of different animal origin, which are commonly used in the leather, upholstery and footwear industries, shall be used.

8.3 Test conditions and incubation period

With the exception of test samples, all laboratory materials shall be autoclaved before use. All test materials, flasks, culture media, etc. shall be autoclaved before use.

The total test time is determined by the time needed for the positive control (culture medium + collagen) to exceed 70 % of the maximum level of biodegradation.

All specimens shall be introduced in powder form. The initial concentration of the specimens shall be between 0,18 g/l to 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).

8.4 Test equipment

8.4.1 Equipment for the assessment of biodegradation by manual titration (equipment A)

The prototype operates in such a way that the generated 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. 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), 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).

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 min^{-1} (to-and-from motion) throughout the entire test duration.

The inoculum volume of each flask varies depending on its degree of activity, changing between 10 % and 20 % of the total volume (inoculum + culture medium), which is 2,6 l.

The air needs to leave the generator through the PSA system which shall have been working for 6 h before the start of the test in order to ensure that a stable CO_2 concentration of less than 1 ppm is achieved in the air flow.

During the test, a constant CO_2-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 CO_2 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 $BaOH_2$ are entered into a spreadsheet that converts them into biodegradation percentages (Clause 11).

8.4.2   Equipment for the assessment of biodegradation by IR detection (equipment B)

The equipment works continuously in a closed system in which the air free of CO_2 circulates throughout the system impelled by a pump situated in the detection equipment (see Figures B.1 to B.5). To increase the amount of oxygen dissolved in the liquid phase, the intake of air into the reaction flask is made through the use of an air diffuser incorporated into the distilling head that is in contact with the liquid medium.

The equipment is provided with a thermostating system capable of regulating the temperature of the reaction flasks by means of an air recirculation system allowing the tests to be carried out at a temperature of 23 °C ± 1 °C. During the whole process, the reaction flasks are constantly agitated (in an orbital system) at 150 min^{-1}.

Before starting the test, all of the detection equipment should be calibrated.

The volume of the inoculum in each flask is 20 % (200 ml) of the total volume (inoculum + culture medium), which amounts to 1,0 l.

The culture medium and the inoculum are added to the flasks and the Erlenmeyer® head with inlet and outlet connectors for the CO_2 detector are installed. Then 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 and aerated for 30 min in all of the flasks with 40 % levels of O_2 (O_2:N_2 mixed at a ratio of 30:70) introducing in the inlet connector a tube connected to a gas mixture cylinder. This procedure is important to remove a small amount of CO_2, produced by the microorganisms resulting from the carbon residues present in the inoculum.

Finally, the nylon tubes are reconnected to the flasks and the test is begun with CO_2 values of 0 %.

The biodegradation equipment features software capable of controlling and recording the values of the CO_2 accumulated during the test at intervals determined by the user.

8.5   End of the test

The test shall be finished, and consequently stopped, when the collagen sample (positive control) has attained the plateau phase with biodegradation values equal to or higher than 70 % of initial carbon.

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

9 Quantification

9.1 Equipment for the assessment of biodegradation by manual titration (equipment A)

9.1.1 Determination of the organic carbon content

Determine by elemental analysis the total organic carbon content of the test material. This allows the theoretical maximum quantity of carbon dioxide evolution to be calculated.

The material has w % (percentage by weight) of carbon;

w/100 • g of material charges = Y g carbon charged to Erlenmeyer® flask

a) $C + O_2 \rightarrow CO_2$

b)   12 g C yield 44 g CO_2

c)   Y mg C yield 44/12 • Y mg CO_2

9.1.2 Determination of the amount of carbon dioxide produced (Method A)

Correct for the amount of carbon dioxide produced with the negative control (culture medium + inoculum) by subtracting negative control titration from test material titration with 0,05 mol/l HCl.

a) $[Ba(OH)_2] + CO_2 \rightarrow BaCO_3 + H_2O$

b) $[Ba(OH)_2] + 2\ HCl \rightarrow BaCl_2 + 2\ H_2O$

c)   m moles of CO_2 = (m moles HCl)/2

9.1.3 Correcting for normality of HCl

a)   m moles of CO_2 = [(0,05 mol/l) • ml HCl]/2

b)   mg of CO_2 = [(0,05 mol/l) • ml HCl • 44]/2 = 1,1 • ml HCl

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

9.1.4 Percentage of biodegradation from carbon dioxide evolved

The percentage of biodegradation from carbon dioxide evolved is calculated as shown below:

a)   (g CO_2 produced/g CO_2 theoretical) • 100

b)   [(1,1 • ml HCl • 12)/(44 • y)] • 100

9.2 Equipment for the assessment of biodegradation by IR detection (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 CO2 that can be generated in each test run to be theoretically calculated.

The material has w % (weight percentage) of carbon;

w/100 • g of sample = Y g of carbon put into the flask

a) $C + O_2 \rightarrow CO_2$

b)   12 g C yield 44 g CO_2

c)   Y g C yield 44/12 • Y g CO_2

9.2.2   Determination of the amount of carbon dioxide (CO_2 produced)

CO_2 produced during the degradation of leather is accumulated in the closed system and measured using the refractive index (RI) present in the quantification equipment. For this purpose, the sensors are previously calibrated between 0 % and 5 % with mixtures of calibration gases. The results obtained are stored in .txt format in a table that can be transformed into an Excel® spreadsheet for the later conversion of % CO_2 to % of biodegradation.

9.2.3   Percentage of biodegradation from CO_2 data

The percentage of biodegradation from carbon dioxide produced is calculated as shown below:

The % of CO_2 produced is converted into a % of biodegradation of the samples from the general equation of the gases. The % CO_2 values obtained in all measuring equipment are mathematically processed to convert them into biodegradation percentages through the general formula of gases ($PV = nRT$), where:

P is the ambient atmospheric pressure on the sample that depends on altitude;

V is the total volume (flasks + tubes + IR detector);

N is the number of moles of the gas mixture;

R is the gas constant;

T is the temperature of the gaseous phase in degrees Kelvin (K).

This formula allows the determination of the number of moles of the gas mixture as a reference for the transformation of CO_2 percentages into % of biodegradation. For such aim, it is necessary to carry out the following mathematical steps:

CO_2 moles $\rightarrow nCO_2 = n$ • % CO_2 (CO_2 moles);

CO_2 mass $\rightarrow nCO_2$ • MCO_2 (g of CO_2);

% Biodegradation $\rightarrow [(MCO_2/MCO_2T)$ • $100]$ (% biodegradation),

where

nCO_2 is the number of CO_2 moles in the gaseous phase.

a)   %CO_2 = CO_2 percentage quantified by the IR equipment at every instant.

b) MCO_2 = CO_2 mass determined for a given % of CO_2 quantified.

c) MCO_2T = Theoretical CO_2 mass obtained from the % of organic carbon in the sample (see 9.2.1).

10 Expression of results

The percentage of degradation of each test material is presented as a relative percentage, calculated on the basis of the absolute value of collagen degradation converted to 100 %, according to Formula (1):

$$X = (B \bullet 100) / A \tag{1}$$

where

A   is the % absolute biodegradation of collagen (70 % or more);

B   is the % absolute biodegradation of each sample;

X   is the % relative biodegradability of each sample.

11 Validity of results

The test shall be considered as valid if the degree of biodegradation of the reference material (collagen) in the reaction flasks is equal to or higher than 70 % in absolute terms (see Clause 8, term A).

12 Test report

The test report shall include at least the following:

a)   a reference to this document, i.e. ISO 20136;

b)   information on the inoculum including source, date of collection, storage, handling and potential acclimation to test material;

c)   carbon (C) content of the test material, both the collagen (positive control) and the leather samples;

d)   percent of theoretical aerobic biodegradation for each leather tested and the standard control material (collagen);

e)   accumulative average carbon dioxide evolution over time until plateau should be reported and displayed graphically as lag-phase and slope (rate);

f)   % relative of biodegradation of each sample.

Annex A
(informative)

Determination of the degree and rate of degradation of the material

A.1 Principle

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, etc.) or polymeric material (plastics), as long as the relevant adjustments are made to the methodology, especially with regards to the inoculum 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 CO_2 throughout the test. For this purpose, the unit complies with strict requirements referring to flow control (CO_2 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
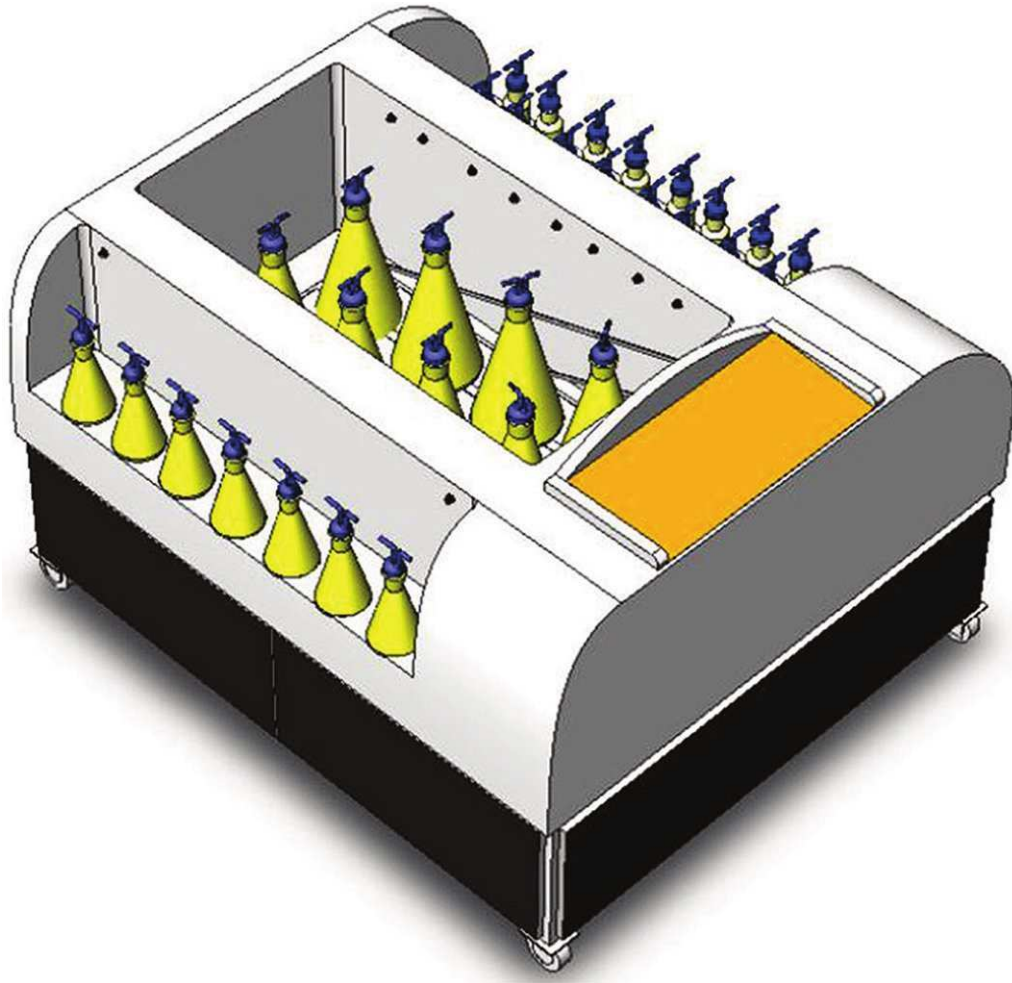
Figure A.2 — Volumetric view of the unit for biodegradability assessment
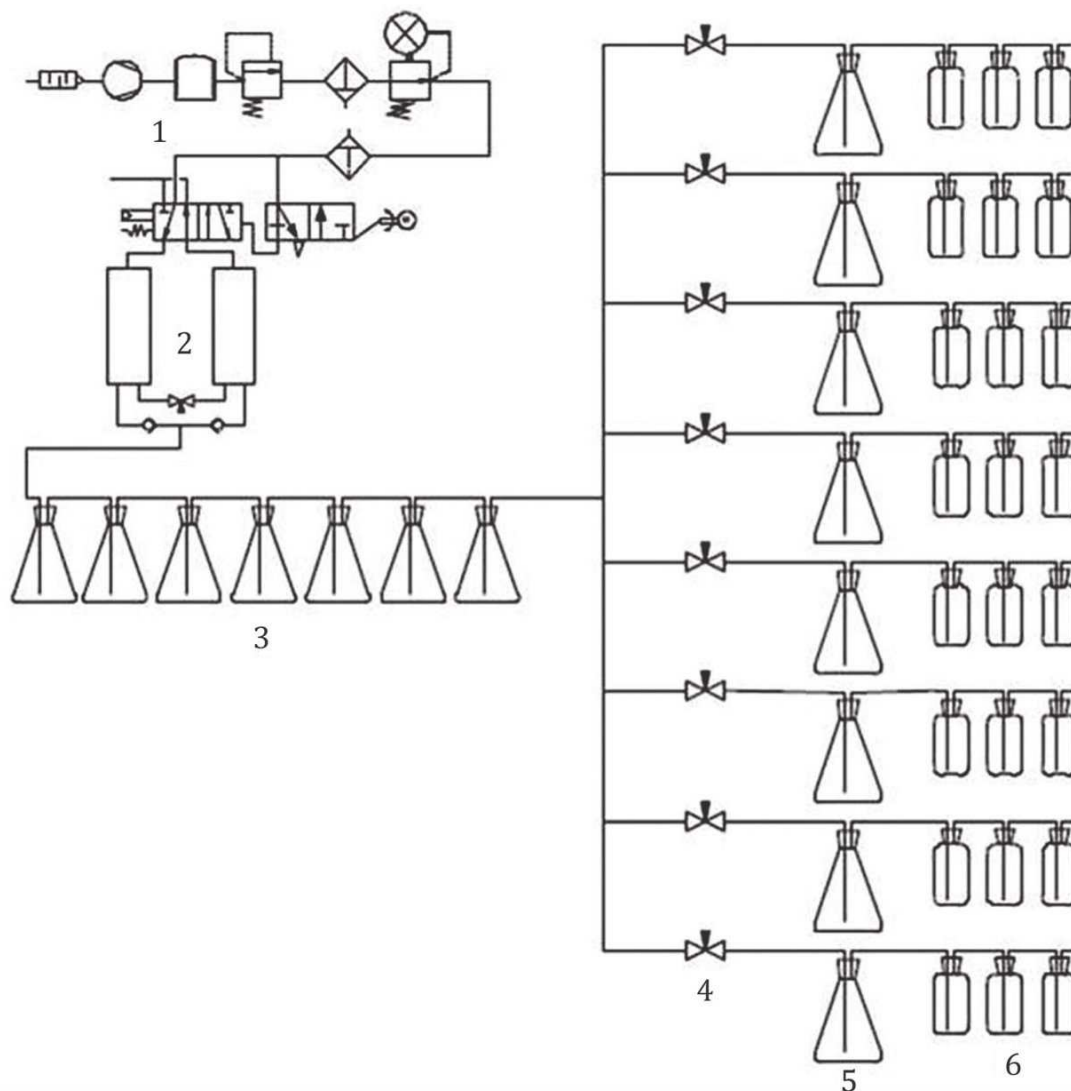
Key

1   air pump
2   PSA system
3   pre-treatment flasks
4   flow meter
5   reaction 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 CO_2-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 CO_2 filter or trap (PSA system). The generated CO_2-free air is bubbled through a series of seven Erlenmeyer® flasks, pre-treatment flasks and this is then divided into eight lines where the flow is 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 Erlenmeyer® flasks, analysis flasks, where the CO_2 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.

Annex B
(informative)

Quantitative determination of leather biodegradation

B.1 Principle

This system comprises an airtight module that incorporates a CO_2 detector based on the detection by differential measurement of IR absorption, being able to measure concentrations of this gas of between 1 % and 5 % to the nearest 0,3 %.

This technique enables great stability of the calibration for long periods of time.
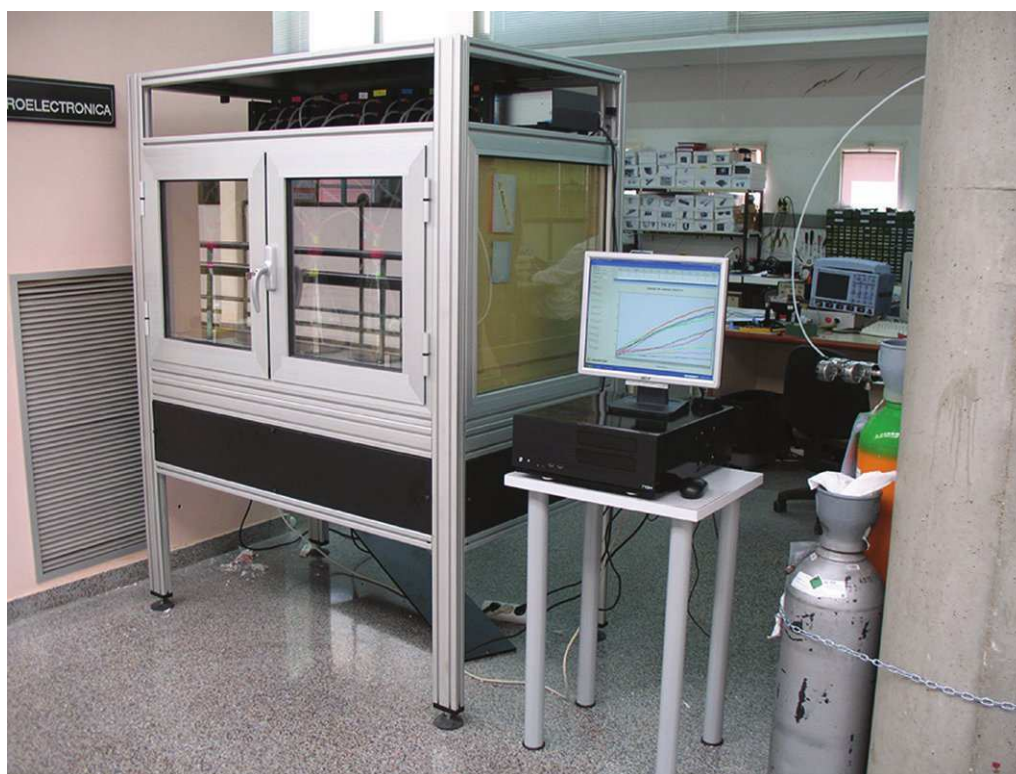
Figure B.1 — General view of the equipment for CO_2 quantification by IR

The CO_2 infrared detection system has the following features.

— It incorporates an air recirculation system based on long-lasting membrane pumps.

— The system can work in both an open and a closed loop.

— In a closed loop, the system allows the recirculation of the aeration gas in biological reaction flasks, and it is possible to monitor the evolution of the reaction directly on the CO_2 concentration,

— in an open loop, the system can capture the gases at the exit of the reactor, provided that the CO_2 concentration generated at a given time is greater than 1 %.

— User-friendly software for signal capture, processing and monitoring, with the capacity to store data for long periods of time.
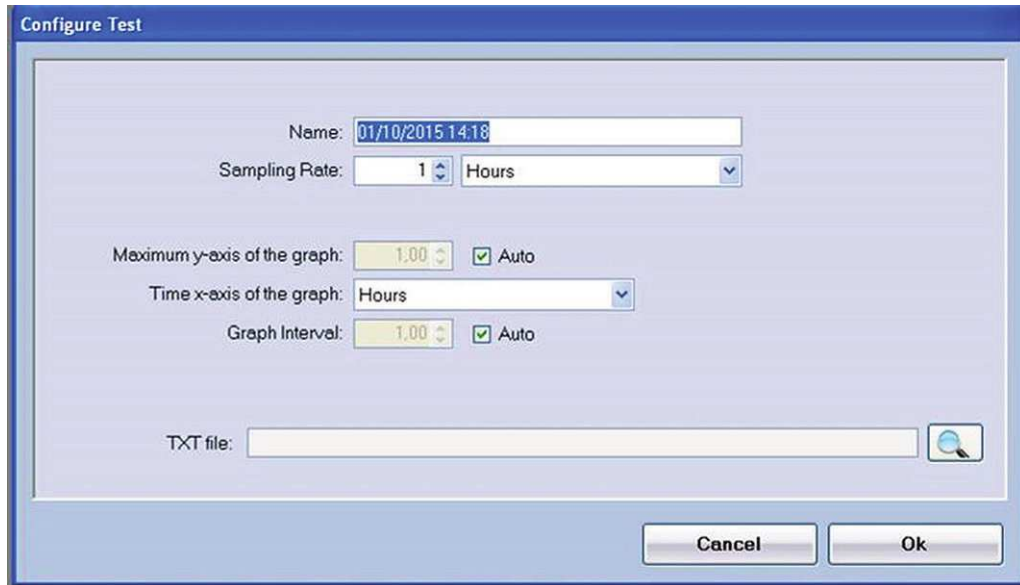
Figure B.2 — Software: test setup screen

— The system incorporates a signal capture and processing unit with which it is possible to control several reaction units simultaneously.

Figure B.3 — Screenshot showing the data stored throughout a test

— The system can be easily calibrated using a standard gas at a known concentration, and it is possible to define the calibration curve with different standard gases at different concentrations.
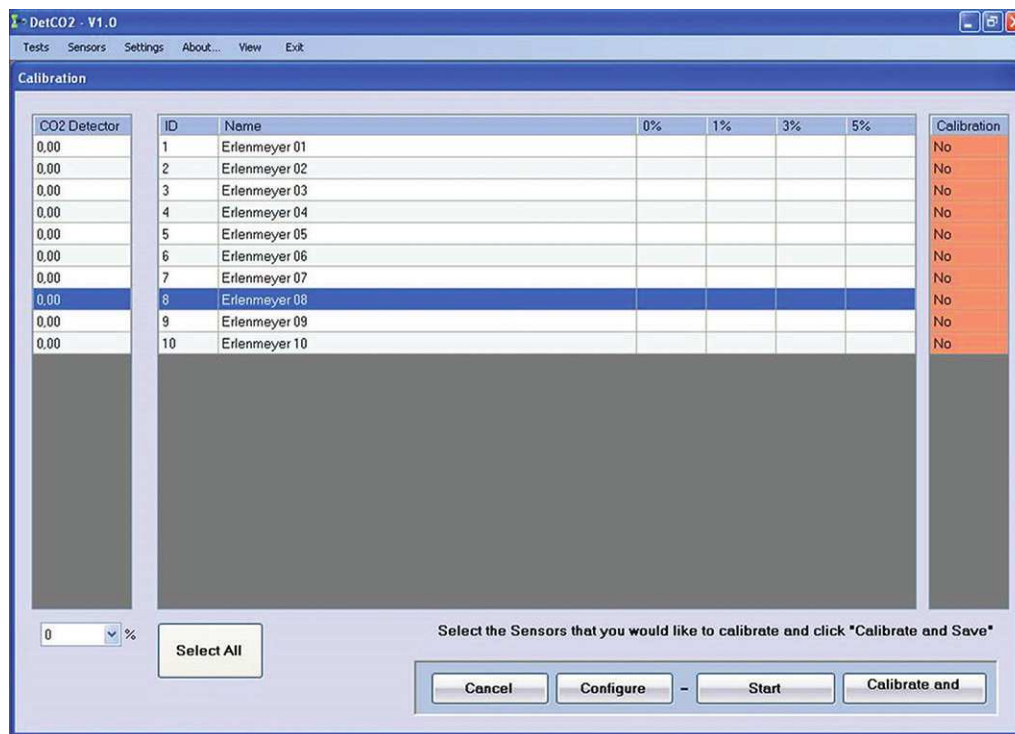
Figure B.4 — Screenshot showing the calibration of the IR sensors

The system is capable of measuring the biodegradation rate of organic polymers, such as leather. The biodegradation process takes place when these materials are exposed to the action of microorganisms, mainly aerobic ones, which are present in a synthetic culture medium where the only carbon and nitrogen source for their growth is found in the leather sample incorporated. The action of microorganisms on this organic matter mainly releases:

— energy for microorganism growth,

— CO_2, and

— H_2O.

Compound + O_2	**microorganisms** $\rightarrow$	**CO_2+H_2O+Biomass**
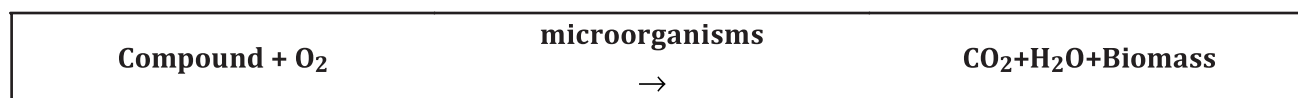
With this system, it is possible to quantify the CO_2 released in the biodegradation process, since the generation of this gas is proportional to the amount of elemental carbon (C_{12}) present in the compound being degraded.

Leather biodegradation can be quantified through the stoichiometry, based on the amount of CO_2 generated by the degradation of the carbon (C_{12}) present in the leather. The studies carried out using this technique employed several modules with their respective reaction flasks into which the compound to be evaluated had been added. With the help of the data capture program, it is possible to determine in which flasks the greatest CO_2 concentration is produced, which will later on provide information about the level of biodegradability. In order to carry out these operations, it is necessary to set a positive control flask, so that all subsequent calculations are made taking this flask as a reference.

The system is conceived to perform tests that can last up to 40 days, with it being able to record all instantaneous values of CO_2 produced, at intervals previously set in the software.
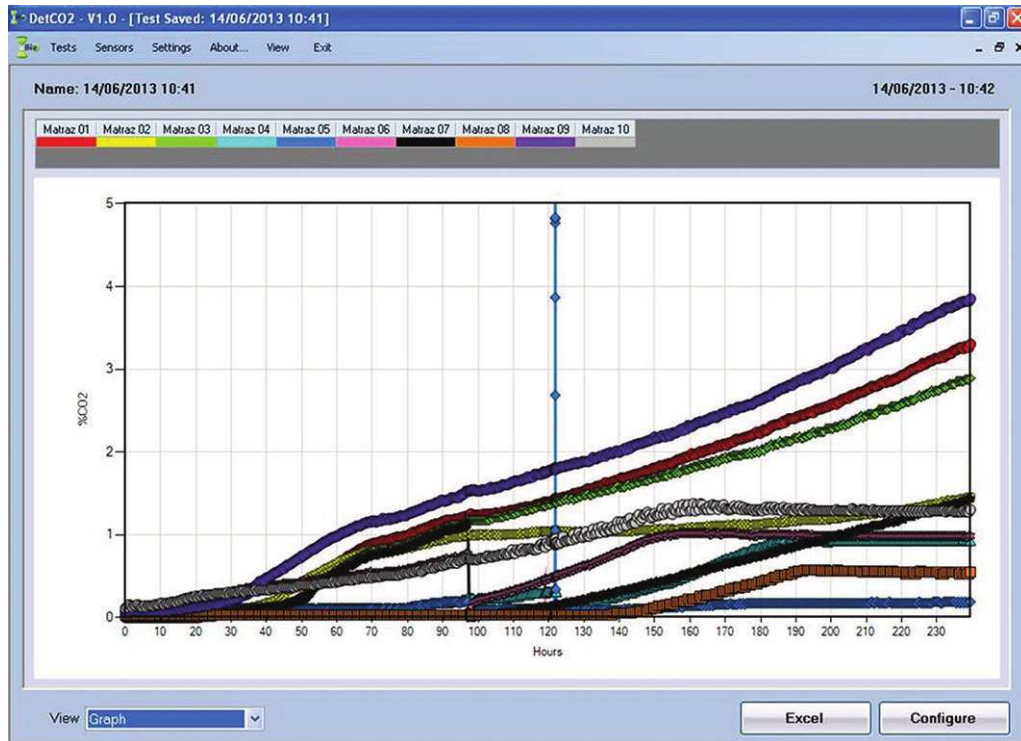
Figure B.5 — Screenshot showing the % of CO$_2$ accumulated in different flasks throughout a test

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