
**Soil quality — Laboratory incubation
systems for measuring the mineralization
of organic chemicals in soil under aerobic
conditions**

*Qualité du sol — Méthodes de mesure de la minéralisation de produits chimiques
organiques dans le sol sous conditions aérobies, au moyen de systèmes
d'incubation de laboratoire*



Contents

Page

1	Scope	1
2	Normative references	1
3	Methods	1
4	Calculation and expresssion of result.....	12
5	Test report.....	13

Annexes

A	Approximate measurement of water-holding capacity of soil.....	14
B	Bibliography	16

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Foreword

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Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

International Standard ISO 14239 was prepared by Technical Committee ISO/TC 190, *Soil quality*, Subcommittee SC 4, *Biological methods*.

Annex A forms an integral part of this International Standard. Annex B is for information only.

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Introduction

This International Standard describes incubation systems for determining the mineralization of organic compounds in soil under aerobic conditions.

Mineralization is only one of the parameters which can be used to assess the biodegradation of organic compounds in soil. If mineralization is not extensive, this does not necessarily mean that the test material is not biodegradable. Material balance studies to assess the production of metabolites, in addition to mineralization studies, provide a comprehensive assessment of biodegradation.

It is essential that this International Standard be used in conjunction with ISO 11266, which gives general guidance on the information needed to assess the potential of an organic compound to be degraded in soil.

Depending on the aim of the study, it is feasible to use a range of incubation conditions, described below, and different methods of analysis.

NOTE — Several trade names of products are given as examples of products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

Soil quality - Laboratory incubation systems for measuring the mineralization of organic chemicals in soil under aerobic conditions

1 Scope

This International Standard specifies three incubation systems for measuring the rates and extent of mineralization of organic compounds in soil by measurement of carbon dioxide evolution. All three incubation systems are applicable to soluble or insoluble compounds but choice of system depends on the overall purposes of the study.

This International Standard does not apply to the use of such systems for material balance studies, which are often test-substance specific.

2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 10381-6:1993, *Soil quality - Sampling - Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in soil.*

ISO 11266:1994, *Soil quality - Guidance on laboratory testing for biodegradation of organic chemicals in soil.*

ISO 11274:¹⁾, *Soil quality - Determination of the water retention characteristic - Laboratory methods.*

3 Methods

3.1 General requirements

The following procedures shall be followed, whichever incubation system is selected.

3.1.1 Soil collection and characterization

Soil shall be collected and handled in accordance with ISO 10381-6. The soil shall be characterized in accordance with ISO 11266:1994, 5.1.1.

¹⁾ To be published.

3.1.2 Test material

The test material shall be characterized in accordance with ISO 11266:1994, 5.2.

3.1.3 Incubation conditions

The following conditions shall be used unless there is a specific reason for using different conditions:

Temperature:	$(20 \pm 2)^{\circ}\text{C}$
Pore water pressure of soil:	-0,01 MPa to -0,03 MPa (measured to $\pm 5\%$) as determined in accordance with ISO 11274 [or between 40% and 60% max. <i>WHC</i> (measured to $\pm 5\%$) in accordance with Annex A.]
Incubation:	in the dark

The incubation conditions should be reported in the test report. If they differ from those above, the reasons for changing them should also be reported in the test report.

A temperature of $(20 \pm 2)^{\circ}\text{C}$ has been chosen as a standard for comparative purposes and because it gives relatively rapid results. Temperatures outside this range can be used if they are more appropriate (for example, because of local conditions, lack of cooling equipment).

3.2 Choice of incubation systems

One of the three systems described in this International Standard, the flow-through system (3.3), the soda-lime column system (3.4) or the biometer system (3.5), shall be used.

Data on the mineralization of organic chemicals can most reliably be obtained from experiments with radiolabelled compounds. Where unlabelled organic chemicals are used, a number of controls are necessary and carbon dioxide evaluation data should be analysed statistically.

Recoveries of carbon dioxide (CO_2) in the three systems can be measured using known quantities of unlabelled or ^{14}C -labelled calcium carbonate and adding sufficient hydrochloric acid to dissolve fully the calcium carbonate.

WARNING The methods in this International Standard use several materials of a hazardous nature. Due care is necessary in their handling and disposal. In particular, all pertinent national regulations should be complied with.

The main advantages and disadvantages of the systems are:

a) Flow-through system:

The main advantages are: sufficient oxygen for long-term, aerobic degradation studies; uses standard laboratory glassware; allows measurement of unlabelled CO_2 (titration), $^{14}\text{CO}_2$ (scintillation counting), and/or ^{14}C -labelled volatile products (scintillation counting).

The main disadvantages are: difficulties with complete recoveries when volatile ^{14}C -compounds are under investigation; sensitivity to leaks in the system.

b) Soda lime column system:

The main advantages are: free access of oxygen for long-term degradation studies; uses standard laboratory glassware; requires little space; adaptable without changes for use with standing or shaken aerobic sediments, pure cultures of microorganisms, algae or plant cell cultures; problem-free incubation under various environmental conditions; full recoveries of applied radioactivity in short- or long-term material balance studies.

The main disadvantages are: $^{14}\text{CO}_2$ trapped in soda lime has to be released and re-adsorbed in liquid for scintillation counting; water content of soils has to be adjusted at least once per month.

c) Biometer system

The main advantages are: requires little space; adaptable without changes for use with standing cultures of aerobic sediments, pure cultures of microorganisms or algae; problem-free incubation under various environmental conditions; ease of measurement of non-radioactive CO_2 (titration), $^{14}\text{CO}_2$ (scintillation counting or ^{14}C -labelled volatile products (scintillation counting).

The main disadvantages are: not ideal for long-term incubations due to lack of free access of air and reduction of partial pressure of oxygen in chamber during incubation; requires special glassware.

3.3 Flow-through system

3.3.1 Principle

This method allows determination of the dissipation and/or metabolism of non-radioactive or ^{14}C -labelled test materials in soil. CO_2 -free air is drawn through the incubation vessel containing the treated soil samples. The CO_2 and organic volatiles evolved from the soil are trapped in a series of absorption traps.

3.3.2 Materials and reagents

Reagents of recognized analytical grade shall be used.

3.3.2.1 Source of CO_2 -free air (e.g. obtained by passing air through an aqueous solution of strong alkali). For studies with ^{14}C -labelled compounds, CO_2 need not be removed from the air unless there is a danger of saturation of the CO_2 traps.

3.3.2.2 Ethylene glycol or ethylene glycol methyl ester, for absorption of organic volatiles.

3.3.2.3 Polyurethane foam trap, density 16 kg/m^3 for absorption of organic volatiles.

3.3.2.4 Sulfuric acid, $c(\text{H}_2\text{SO}_4) = 0,5 \text{ mol/l}$, for absorption of alkaline volatiles.

3.3.2.5 Sodium or potassium hydroxide solution, $c(\text{KOH})$ [or (NaOH)] = $0,1 \text{ mol/l}$ to $0,5 \text{ mol/l}$ for absorption of nonradioactive CO_2 ; or scintillation cocktail for absorption of $^{14}\text{CO}_2$ ¹⁾.

1) Carbosorb (Canberra Packard) and Oxisolve (Zinsser) are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

WARNING If the scintillation cocktail is used as a trap, volatile organic amines and solvents can accumulate in toxic concentrations and there is danger of explosion. Therefore it is essential that the work area is well ventilated.

3.3.2.6 Scintillation cocktails for determination of the $^{14}\text{CO}_2$ in alkali traps¹⁾.

3.3.3 Apparatus and glassware

3.3.3.1 Liquid scintillation counter

3.3.3.2 Scintillation vials

3.3.3.3 Temperature-controlled incubator or room ($\pm 2^\circ\text{C}$)

3.3.3.4 Membrane pump (capacity, approximately $2,8 \text{ m}^3/\text{h}$)

3.3.3.5 Flow meter

3.3.3.6 Flow-restrictor valves

3.3.3.7 Glass dishes for system I, e.g. moist soil (equivalent to 50 g dry mass)

- diameter 5 cm, height 5 cm - for samples equivalent to 50 g air-dried soil

- diameter 9,5 cm, height 5 cm - for samples equivalent to 300 g air-dried soil

3.3.3.8 Erlenmeyer flask (250 ml) for system II

3.3.3.9 Gas washing bottles (100 ml) for absorption traps

3.3.3.10 Gas washing bottles (200 ml to 500 ml) for moistening the air.

3.3.4 Procedure

Choose incubation system I or II described below. System I is more applicable when many samples have to be incubated in limited space, system II requires more space but is applicable for small-scale experiments.

3.3.4.1 Incubation system I

Incubation of soil samples shall take place in temperature-controlled incubators or rooms (3.3.3.3.). Set up cylindrical, separately removable incubation units in the chamber (see Figure 1). The incubation units shall contain sets of soil samples in glass dishes (3.3.3.7) (normally one incubation set consists of 6 sub-samples). Each incubation unit can be aerated separately.

In order to ensure aerobic conditions, draw a constant stream of CO_2 -free air (3.3.2.1) through each incubation unit using a membrane pump (3.3.3.4).

3.3.4.2 Incubation system II

Incubate the soil sample in a glass flask (e.g. Erlenmeyer flask) (3.3.3.8) in a temperature-controlled room or incubator (3.3.3.3). Draw a constant stream of CO_2 -free air (3.3.2.1) through the flask.

1) Hionic fluor and Optifluor (Canberra Packard) are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

3.3.4.3 Absorption of volatile products

For both systems, moisten the CO₂-free air passing over the soils by bubbling it through 2 gas wash bottles (3.3.3.1) about half-filled with acidified, deionized water (approximately 1 ml of concentrated sulfuric acid per litre of water). Distribute the water-saturated air to the different incubation units via valves (3.3.3.6).

Establish a constant flow of approximately 0,1 l/min through each incubation unit; use a flow meter (3.3.3.5) to measure the flow rates.

For both systems, bubble the outgoing gas through an absorption system to capture volatilized parent compound, volatile metabolite, and CO₂ for subsequent analyses. All connections shall be made of stainless steel or polytetrafluoroethylene (PTFE) tubing. Quantify any ¹⁴C-labelled compounds by liquid scintillation counting, as appropriate.

The absorption systems consist of:

- one gas washing bottle (3.3.3.9) filled with reagent for absorption of organic volatiles (3.3.2.2 or 3.3.2.3);
- one gas washing bottle (3.3.3.9) filled with reagent for absorption of alkaline volatiles (3.3.2.4) (if necessary);
- one gas washing bottle for absorption of CO₂ (3.3.2.5). If high rates of CO₂-production are expected, a second CO₂ trap is needed.

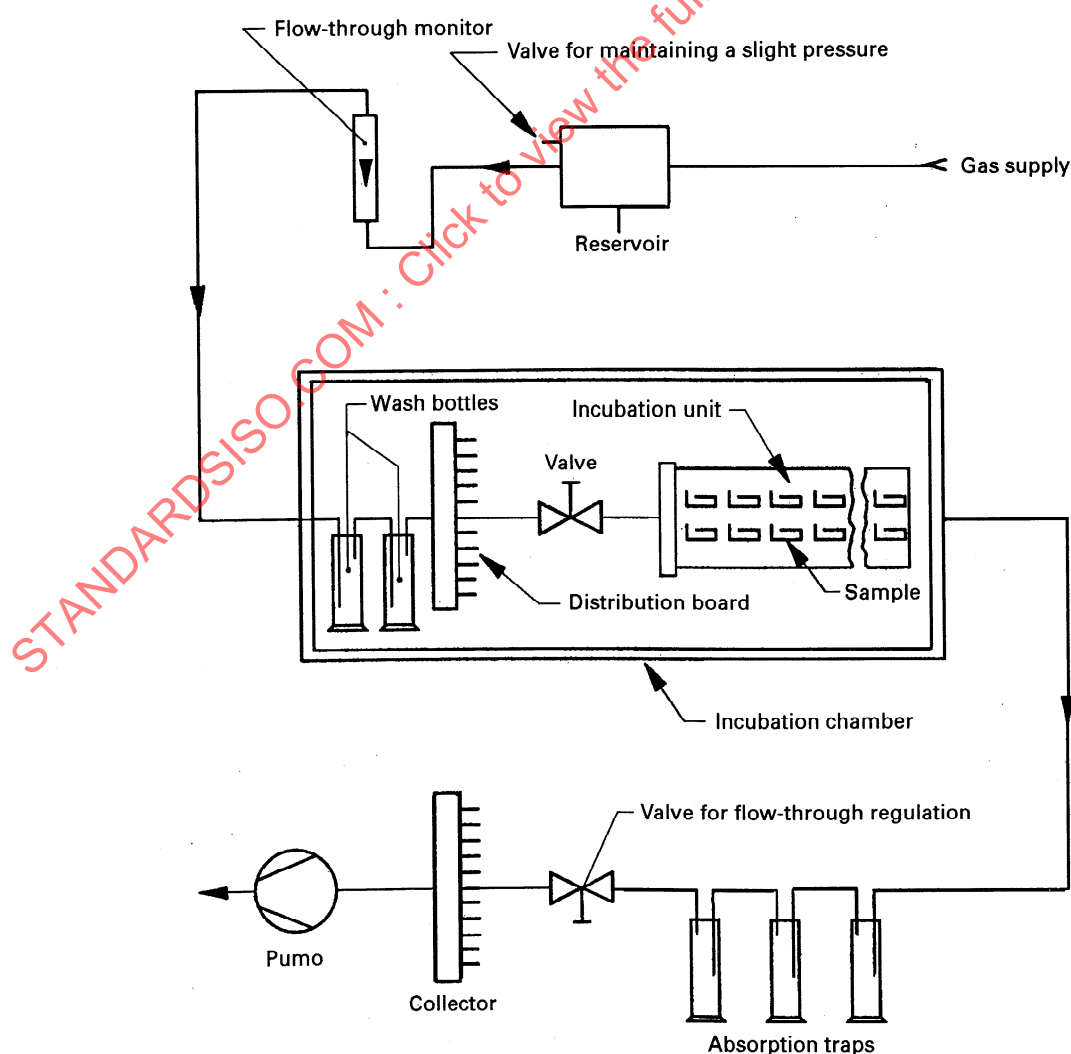


Figure 1 – Flow-through incubation system

3.4 Soda-lime column system

3.4.1 Principle

This system allows determination of the dissipation and/or metabolism of ^{14}C -labelled test materials in soil. Soil treated with the ^{14}C -labelled test materials is held in a flask with a ground-glass jointed neck into which a ground-glass jointed glass column has been inserted (see figure 2). The glass column contains a trap for volatilized ^{14}C -labelled materials and a trap for $^{14}\text{CO}_2$. Oxygen and atmospheric gases other than CO_2 move freely into and out of the flask by diffusion.

NOTE In addition to use with soils, the system has also been used for aerobic degradation studies with standing or shaken sediments, pure cultures of microorganisms, algae and plant cell cultures.

3.4.2 Materials and reagents

Reagents of recognized analytical grade shall be used.

3.4.2.1 Granulated soda lime, mesh size 1,5 mm to 3 mm, containing a saturation indicator

3.4.2.2 Glass wool

3.4.2.3 Paraffin oil solution in hexane (2% V/V) for coating glass wool plugs with oil

3.4.2.4 Hydrochloric acid (HCl) (ca. 18% V/V) for dissolution of soda lime granules

3.4.2.5 Carbon dioxide-absorbing solution; e.g. 1 mol/l NaOH (see figure 3) or other suitable CO_2 -trapping solutions¹⁾ (see figure 4).

3.4.2.6 Scintillation cocktail suitable for mixing with NaOH (if applicable)

3.4.3 Apparatus and glassware

3.4.3.1 Liquid scintillation counter

3.4.3.2 Scintillation vials

3.4.3.3 Temperature-controlled incubator or room ($\pm 2^\circ\text{C}$)

3.4.3.4 Nitrogen gas

3.4.3.5 Flow meter

3.4.3.6 Flow restrictor valves, if required for the glassware set-up for CO_2 evolution.

3.4.3.7 Erlenmeyer flask (e.g. 300 ml) with a ground-glass jointed neck (e.g. 24 or 29 standard joint)

3.4.3.8 Open-ended glass tube (reflux column) fitted with a ground-glass standard joint at one end (e.g. 24 or 29); length about 13 cm, diameter about 1,5 cm to 2 cm (figure 2).

3.4.3.9 Glassware and equipment for transferring the $^{14}\text{CO}_2$ bound by the soda lime (figure 3 or 4) to an absorbent (3.4.2.5) that is compatible with the scintillation cocktail (3.4.2.6).

1) An example of a suitable solution is a 5:4 V/V mixture of Permafluor with Carbosorb (Canberra Packard). This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

3.4.4 Procedure

3.4.4.1 Preparation of column for trapping ^{14}C -labelled organic materials and $^{14}\text{CO}_2$

Prepare a glass column (3.4.3.8) that serves as the extended neck of the incubation flask and which holds, from the base upward, an oil-coated glass wool plug, (which serves as a trap for volatilized ^{14}C -labelled organic materials), and 8 g to 10 g of soda lime, (which serves as a trap for $^{14}\text{CO}_2$) (figure 2). Coat the glass wool plugs with oil by dipping them in an oil-hexane solution (3.4.2.3) and allowing the hexane to evaporate under a fume hood. For experiments that last for more than 1 month, use an additional plug and layer of soda lime to protect the $^{14}\text{CO}_2$ trap from saturation with atmospheric CO_2 , see figure 2.

3.4.4.2 Incubation of soil treated with ^{14}C -labelled test material

Place soil treated with the ^{14}C -labelled test chemical into the Erlenmeyer flask (3.4.3.7), and close the neck of the flask with the prepared glass column (3.4.4.1). The soda lime on top of the plugs serves as the trap for $^{14}\text{CO}_2$. Soda lime can chemically bind up to 20% of its mass in CO_2 , however, if the self-indicating soda lime granules change colour, replace them with fresh soda lime. There is no danger of loss of $^{14}\text{CO}_2$ from the soda lime so it can be stored in closed containers until analysis. Incubate the soil samples in the temperature-controlled room or incubator (3.4.3.3).

3.4.4.3 Transfer of $^{14}\text{CO}_2$ from soda lime to a scintillation cocktail

Carry out this process in a fume cupboard. Pour the soda lime granules from the $^{14}\text{CO}_2$ trap into a vacuum flask and assemble the glassware as shown in figure 3, figure 4 or any other equivalent glassware. Add 50 ml of hydrochloric acid (3.4.2.4) dropwise to the soda lime while maintaining a slow flow of nitrogen gas (3.4.3.4) (e.g. 2 l/h to 5 l/h) through the system to sweep the liberated $^{14}\text{CO}_2$ into the CO_2 -absorbing solution. Maintain the temperature of the water bath at 60°C to 70°C.

After the soda lime has fully dissolved, sweep the system with a stream of the nitrogen gas for at least 20 min to ensure transfer of all traces of $^{14}\text{CO}_2$ from the flask into the CO_2 trapping devices. Take samples (figure 3) or the whole trap (figure 4) for liquid scintillation counting (3.4.3.1), after mixing with a scintillation cocktail (3.4.2.6) if needed.

NOTE If NaOH is used to capture $^{14}\text{CO}_2$, it is possible to determine whether the radioactivity consists exclusively of $^{14}\text{CO}_2$ or a mixture including ^{14}C volatile organics. $^{14}\text{CO}_2$ can be eliminated from a sample of the NaOH by slowly acidifying to ~ pH 1. The acidified solution can then be measured for loss of radioactivity.

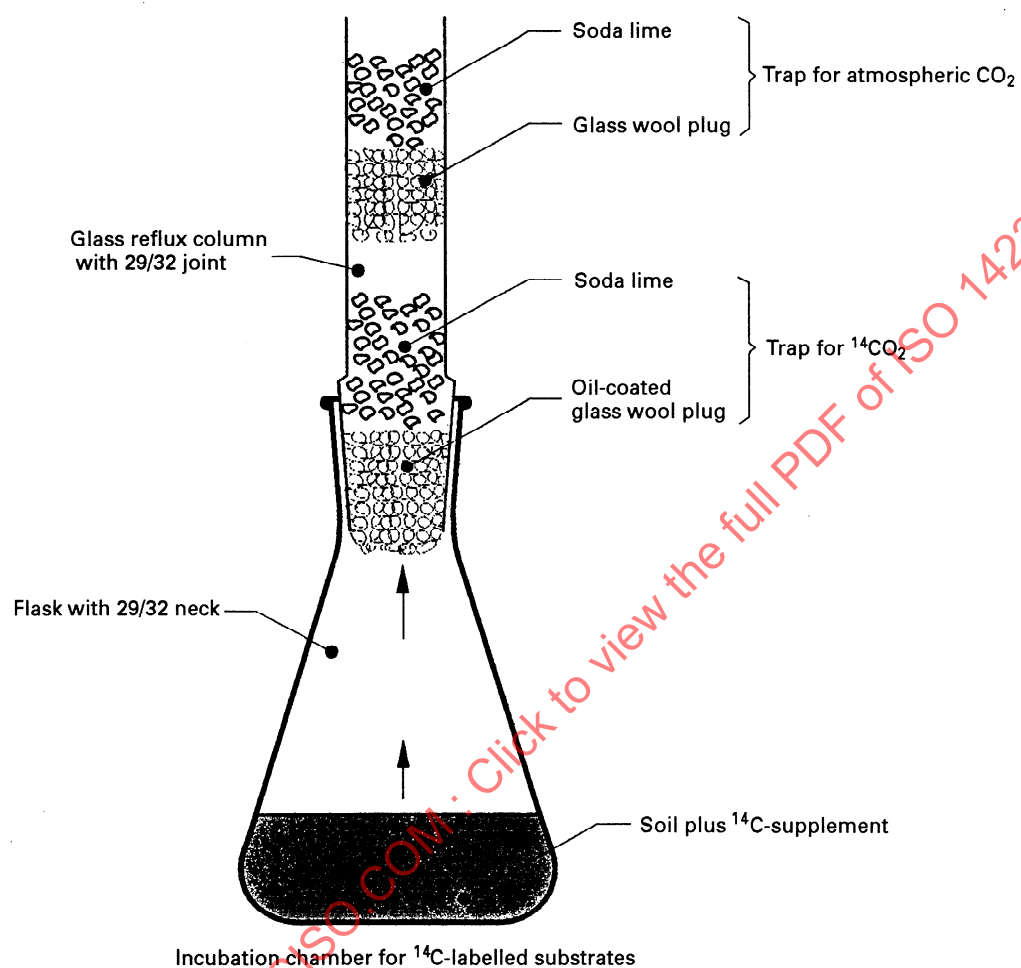


Figure 2 – Incubation vessel for aerobic soil metabolism

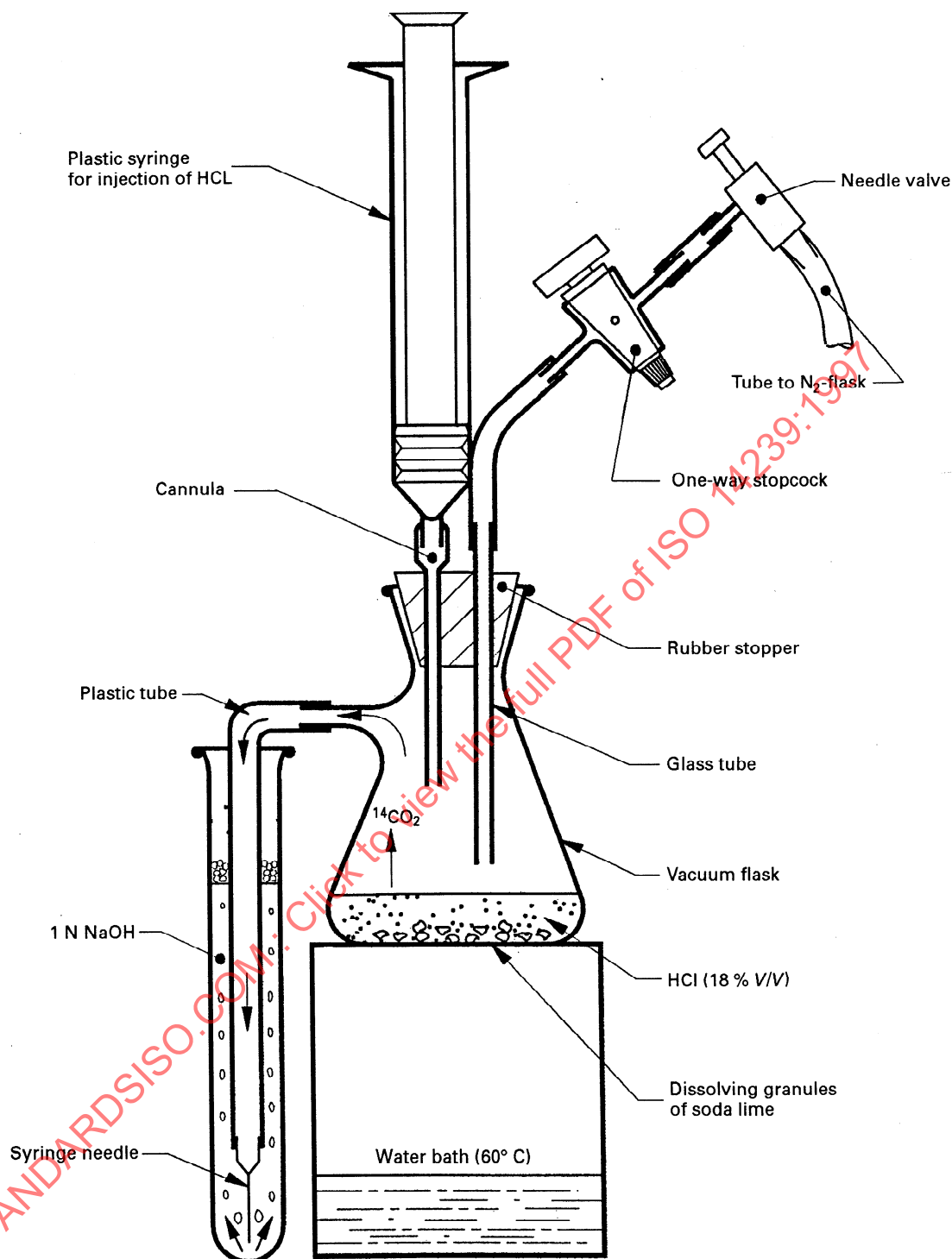


Figure 3 – Example of apparatus for the liberation of bound $^{14}\text{CO}_2$ from soda lime and its quantitative recapture in 1 M NaOH

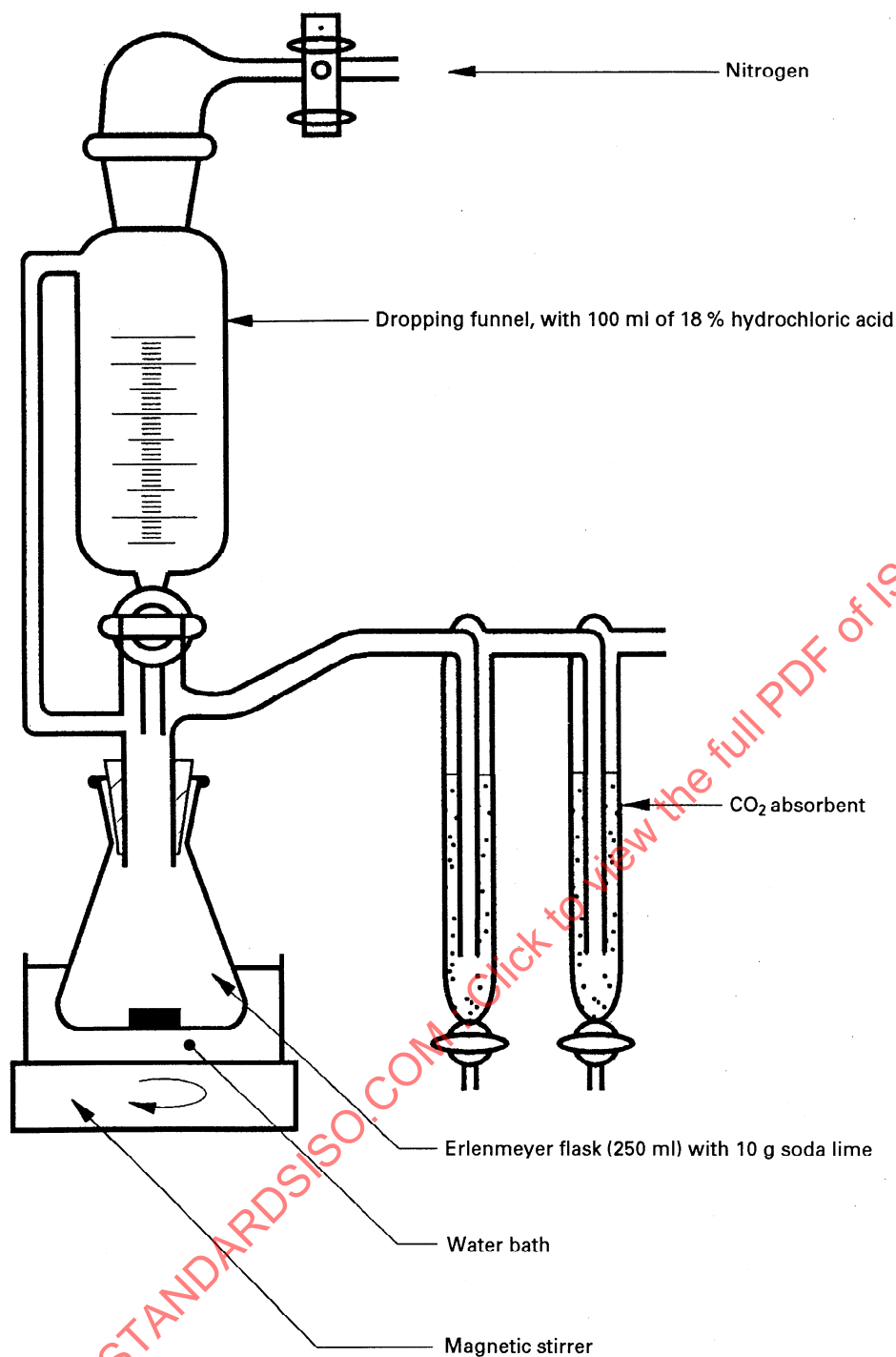


Figure 4 – Example of apparatus for the liberation of bound $^{14}\text{CO}_2$ from soda lime and its quantitative recapture in scintillation liquid containing a CO_2 absorbing liquid

3.5 Biometer system

3.5.1 Principle

Soil treated with the non-radioactive- or ^{14}C -labelled test material is poured into the main chamber of the biometer flask (see figure 5). The CO_2 or $^{14}\text{CO}_2$ released during degradation of the chemical is captured in the aqueous alkali in the side-arm of the flask. Quantitative analyses for non-radioactive CO_2 trapped by the alkali are made using classical titration procedures. Analyses for $^{14}\text{CO}_2$ are made by liquid scintillation counting.

3.5.2 Materials and reagents

3.5.2.1 Potassium or sodium hydroxide solution, $c(\text{KOH})$ [or (NaOH)] = 1 mol/l.

3.5.2.2 For studies with non-radioactive test materials: **soda lime or other CO_2 -absorbing material**¹⁾

3.5.2.3 For studies with ^{14}C -labelled test materials: **scintillation cocktails** for the determination of $^{14}\text{CO}_2$ captured in the alkali trap²⁾

3.5.3 Apparatus and glassware

The following is required:

3.5.3.1 250 ml Erlenmeyer flask with a side-arm made from a 50 ml round-bottom glass tube fused to it (figure 5)

3.5.3.2 25 ml syringes (e.g. Luer-lock)

3.5.3.3 Syringe needle (15 gauge, 15 cm long)

3.5.3.4 Pipettes and measuring cylinders

3.5.3.5 Temperature controlled incubator or room ($\pm 2^\circ\text{C}$)

In addition, for studies with ^{14}C -labelled test materials:

3.5.3.6 Liquid scintillation counter

3.5.3.7 Scintillation vials

1) Ascarite (Aldrich Chemical Co. Ltd.) is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

2) Hionic fluor and Optifluor (Canberra Packard) are examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products.

3.5.4 Procedure

Treat the soil sample with the test chemical, place into the main chamber of the biometer flask (3.5.3.1), and seal with a gas-tight stopper. For tests with non-radioactive test material, insert a CO₂-absorbing filter into the stopper (see figure 5). This filter shall consist of a tubular funnel, filled with CO₂-absorbing material (3.5.2.2) and fitted with a rubber stopper at the larger end and a stopcock at the narrower end. Partially fill the side-tube of the biometer with 10 ml of alkali (3.5.2.1) and seal it. Incubate the biometer flask and its contents as described in 3.3. Remove the alkali in the side arm at intervals for analysis by titration for non-radioactive CO₂, and by liquid scintillation counting for ¹⁴CO₂.

For tests with non-radioactive test materials, avoid introduction of exogenous CO₂ by removing the alkali using a hypodermic needle (figure 5). Close the end of the needle exposed to the atmosphere by an air-tight rubber stopper, and cover the tip of the needle, which is immersed in alkali, with a short length of silicone tubing. During removal of alkali, remove the stopper on the CO₂-absorbing filter and open the stopcock to allow access of CO₂-free air.

Before introducing fresh alkali into the side-arm, aerate the system, if necessary. This is done by drawing air through the system via the CO₂-absorbing filter.

NOTE To ensure that the radioactivity in the alkali used to capture the ¹⁴CO₂ consists exclusively of ¹⁴CO₂, a sample of the alkali can be carefully and slowly acidified to about pH 1 to remove ¹⁴CO₂ and then checked for the presence of residual radioactivity. This operation should be carried out under a fume hood.

4 Calculation and expression of results

4.1 For unlabelled test materials

At each sampling point calculate the amount of CO₂ trapped in the alkali taking into account any dilutions prior to analysis. Subtract the amount of CO₂ found in untreated soil controls (this represents normal respiration activity of soil micro-organisms). Compare this background-adjusted figure for CO₂ production with the total theoretical CO₂ obtained from the complete oxidation of the carbon added to the incubation assembly as test material:

$$\text{e.g. } \frac{\text{CO}_2 \text{ produced at sampling point 1}}{\text{Theoretical CO}_2 \text{ from test material}} \times 100 = \% \text{ mineralization}$$

Calculate the cumulative % mineralization for each sampling point (SP) i.e. cumulative % mineralization at SP 3 = (CO₂ at SP 1) + (CO₂ at SP 2) + (CO₂ at SP 3). Present the cumulative % mineralization in tabular and graphical forms.

4.2 For ¹⁴C-labelled test materials

At each sampling point calculate the amount of ¹⁴CO₂ trapped in alkali by liquid scintillation counting taking into account any dilutions prior to analysis. Compare this with the total ¹⁴C added to the incubation assembly as test material:

$$\text{e.g. } \frac{{}^{14}\text{CO}_2 \text{ produced at sampling point 1}}{{}^{14}\text{C from test material}} \times 100 = \% \text{ mineralization}$$

Calculate the cumulative % mineralization for each sampling point (SP), i.e. cumulative % mineralization at SP 3 = (CO₂ at SP 1) + (CO₂ at SP 2) + (CO₂ at SP 3). Present the cumulative % mineralization in tabular and graphical forms.

5 Test report

Report the details for characterizing the soil and the test material in accordance with ISO 11266:1994, clause 9.

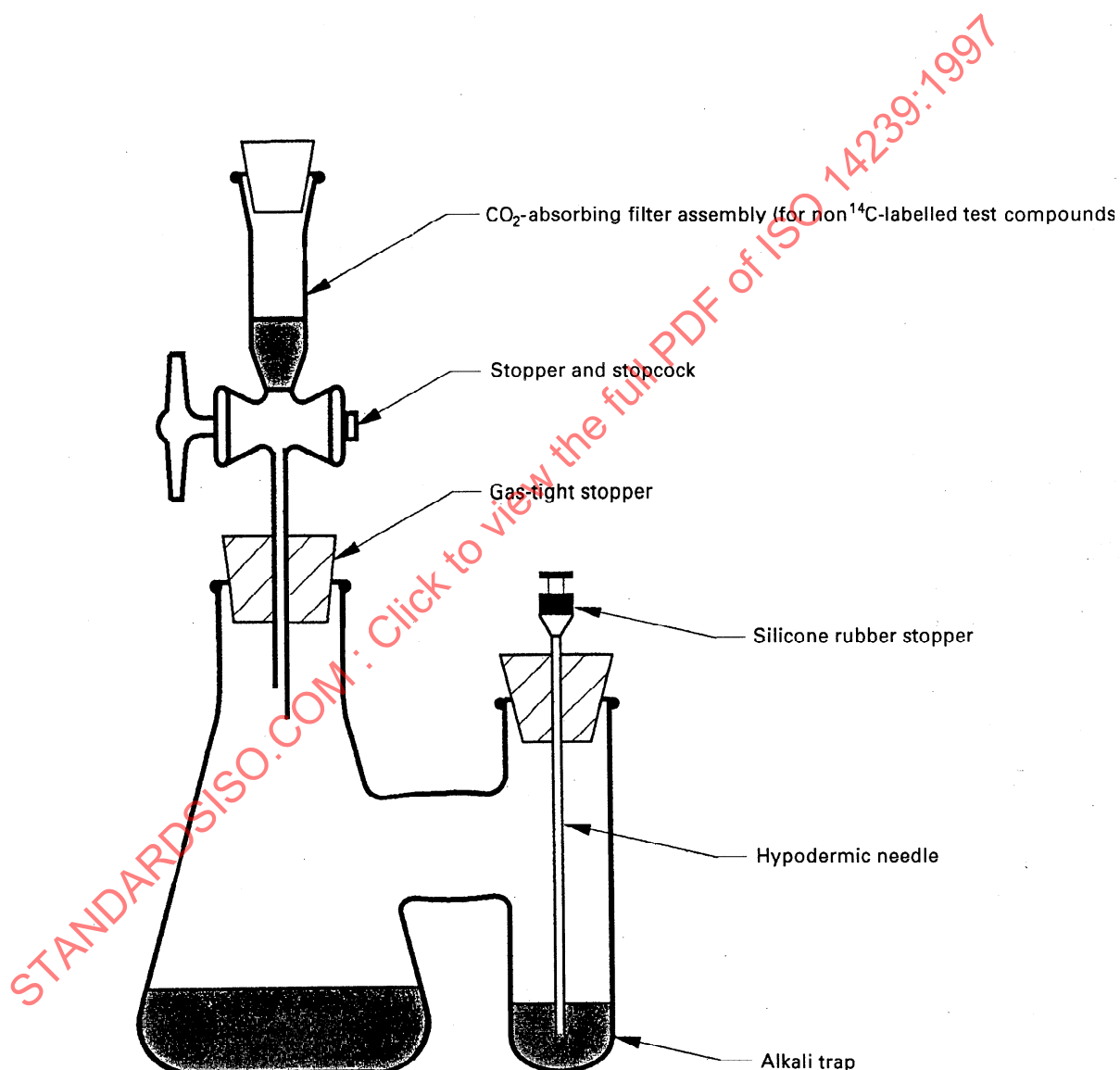


Figure 5 – Biometer flask

Annex A

(normative)

Measurement of water-holding capacity of soil

A.1 Scope

The method is applicable for obtaining a value for water-holding capacity for applications in which the exact value is not critical.

A.2 Principle

A cylinder with a perforated base is partially filled with soil, capped and immersed in water and drained. The quantity of water taken up by the soil is determined by weighing, drying to constant mass at 105°C and reweighing.

A.3 Apparatus

A.3.1 Cylinder, of known volume, of approximate dimensions 50 mm to 150 mm in length and 50 mm to 100 mm in diameter, with a perforated base.

A.3.2 Water bath (room temperature)

A.3.3 Tray, with a drainage hole, containing wet fine quartz sand to a depth of 20 mm to 50 mm.

A.3.4 Oven, capable of maintaining a temperature of 105°C to within ± 2 °C.

A.3.5 Balance, capable of weighing to an accuracy of $\pm 0,01$ g.

A.4 Method

Cover the perforated base of the cylinder (A.3.1) with a filter paper and weigh the cylinder with the filter paper. Partially fill the cylinder with soil and cap the cylinder. Submerge the cylinder in a water bath until the water level is above the top of the soil. Incubate for 2 h at room temperature. Remove the cylinder from the water and place the cylinder on a tray of sand (A.3.3) and allow to drain for 2 h to 24 h, depending on the soil type. Weigh the cylinder with soil, remove the soil and dry it to constant mass at 105 °C, and weigh the soil again.

A.5 Calculation

Calculate the water-holding capacity, *WHC*, as follows:

$$WHC = \frac{S - T - D}{D} 100\%$$

where *WHC* = Water-holding capacity, in percent

S = Mass of water-saturated soil + cylinder + filter paper, in grams

T = Tare (mass of cylinder + filter paper), in grams

D = Dry mass of soil, in grams

A.6 Expression of results

Express the water-holding capacity of the soil (*WHC*) as a percentage of the dry mass of soil.

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