SLOVENSKI STANDARD
SIST ISO 10260:2001

01-december-2001

Kakovost vode - Merjenje biokemijskih parametrov - Spektrometriļ no doloļ evanje koncentracije klorofila

Water quality -- Measurement of biochemical parameters -- Spectrometric determination of the chlorophyll-a concentration

Qualité de l'eau -- Mesurage des paramètres biochimiques -- Dosage spectrométrique de la chlorophylle a

Ta slovenski standard je istoveten z: **ISO 10260:1992**

ICS:

13.060.50	Preiskava vode na kemiļ ne snovi	Examination of water for chemical substances

SIST ISO 10260:2001 **en**

INTERNATIONAL STANDARD

ISO 10260

First edition
1992-07-15

Water quality — Measurement of biochemical parameters — Spectrometric determination of the chlorophyll-a concentration

Qualité de l'eau — Mesurage des paramètres biochimiques — Dosage spectrométrique de la chlorophylle a

Reference number
ISO 10260:1992(E)

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.

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 10260 was prepared by Technical Committee ISO/TC 147, *Water quality*, Sub-Committee SC 2, *Physical, chemical, biochemical methods*.

Annexes A and B of this International Standard are for information only.

International Organization for Standardization
Case Postale 56 • CH-1211 Genève 20 • Switzerland

Printed in Switzerland

Introduction

Chlorophyll-a is the essential photosynthetic pigment present in all green plants. The chlorophyll content of a surface water is an indicator of its trophic state. The determination of the chlorophyll-a concentration provides information concerning the quantity and potential photosynthetic activity of algae. The most important metabolites of chlorophylls are phaeophytines and phaeophorbide. The ratio of chlorophyll to phaeopigments is indicative of the physiological state of the algae.

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Water quality — Measurement of biochemical parameters — Spectrometric determination of the chlorophyll-a concentration

1 Scope

1.1 This International Standard specifies a method for the determination of the chlorophyll-a concentration. The procedure can be applied for phytoplankton in natural surface waters and for testing algal growth in bio-assays. Using appropriate sampling it can also be applied to phytobenthic communities (see annex A).

1.2 Other algal pigments such as chlorophyll-b and chlorophyll-c and some chlorophyll metabolites do not contribute to the determination. Phaeopigments may be determined semiquantitatively, to correct for interference with chlorophyll-a determination and to indicate the portion of inactive algal biomass.

1.3 Chlorophyll is sensitive to light and oxygen, especially when it is extracted. To avoid oxidative and photochemical destruction, the samples shall not be exposed to bright light or air. Homogenization of the sample may in some cases increase the extraction efficiency.

1.4 The extraction procedure with ethanol involves heating to 75 °C for 5 min to inactivate chlorophyllase and accelerate the lysis of pigments. Storage of extracts (except filters containing suspended matter) prior to photometric measurement should be kept to a minimum, but is possible up to 3 d under refrigeration at 4 °C. Storage of extracts at less than − 25 °C is possible for at least 30 d.

1.5 Even though the procedure involves filtration or centrifugation to clarify the final extract, a slight turbidity may remain. The acidification step may also cause turbidity. Therefore, the absorbance measured at 665 nm has to be corrected for turbidity by substracting the absorbance measured at 750 nm.

1.6 The pigment of certain rarely occurring phototrophic bacteria (e.g. *Chlorobium*) interferes with the determination of chlorophyll-a concentration [1]. The contribution of chlorophyll-b and chlorophyll-c to the absorbance at 665 nm is negligible [2].

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 to 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 5667-1:1980, *Water quality — Sampling — Part 1: Guidance on the design of sampling programmes.*

ISO 5667-2:1991, *Water quality — Sampling — Part 2: Guidance on sampling techniques.*

3 Principle

Collection of algae and other suspended matter from a water sample by filtration. Extraction of algal pigments from the filter residue into hot ethanol. Spectrometric determination of the chlorophyll-a concentration in the extract. Evaluation of the chlorophyll-a and phaeopigment concentration from the difference in absorbance at 665 nm prior to and after acidification of the extract [3] [4].

4 Reagents

Use only reagents of recognized analytical grade and only deionized water of equivalent purity.

4.1 Hydrochloric acid, $c(HCl) = 3$ mol/l.

4.2 Ethanol,
(C_2H_5OH), aqueous solution 90 % (V/V).

NOTE 1 Generally, a denaturant in ethanol does not interfere. Nevertheless, a comparative determination with pure ethanol (90 %) is recommended with each unknown batch [4].

5 Apparatus

Ordinary laboratory apparatus and the following:

5.1 Spectrometer, for use in the visible range up to 750 nm, with a resolution of 1 nm, a bandwidth of 2 nm or less, sensitivity less than or equal to 0,001 absorbance units and with optical cells of path length between 1 cm and 5 cm.

5.2 Vacuum filtration device, filter holder with clamp.

5.3 Glass-fibre filters free of organic binder, for filtration of water samples, retaining more than 99 % of particles greater than 1 µm. Suitable diameters range from 25 mm to 50 mm.

5.4 Filters for filtration of extracts, as described in 5.3 but of small diameter e.g. 25 mm.

Alternative: Centrifuge, with an acceleration of 6 000 g and a rotor suitable for appropriate extraction tubes.

5.5 Water bath, adjustable to 75 °C ± 1 °C with a rack for extraction vessels.

5.6 Extraction vessels, e.g. wide-necked amber glass vials with polytetrafluoroethylene (PTFE)-lined screw caps, of typical capacity 30 ml to 50 ml, suitable for centrifugation at 6 000 g.

6 Sampling and storage

Sample according to ISO 5667-1 and ISO 5667-2. Refrigerated storage of water samples in the dark for less than 8 h is acceptable but should be avoided. If possible, perform steps 7.1 to 7.3 immediately after sampling. If necessary, store the raw extracts in air-tight brown glass extraction vessels (5.6) below − 25 °C for up to 30 d. Do not store frozen water samples or filters plus solids.

7 Procedure

7.1 Filtration

Shake the samples in order to mix thoroughly. Filter a measured volume of sample V_s (normally in the range 0,1 litre to 2 litres, depending on the concentration of algae) through a glass-fibre filter (5.3) clamped in a suitable holder. Dry the filter in a vacuum, as soon as it is dry remove it from the holder and place it in the extraction vessel. If it does not fit into the extraction vessel, tear it into pieces.

Avoid contact with fingers.

7.2 Extraction variant A

Heat the required volume of ethanol (4.2) to 75 °C.

Pour a small volume (usually 30 ml to 40 ml) of the hot ethanol into the vessel containing the filter or filter pieces. After cooling for a few minutes, grind the filter to facilitate extraction, preferably with a rod-shaped homogenizer. Wash the homogenizer rod with a small volume of ethanol (4.2) to remove particles of sample adhering to it. Extract the suspension for at least 3 min.

NOTE 2 Usually the extraction is carried out at room temperature for several hours or overnight. If the extraction is prolonged, or the extract is stored for several days (e.g. over the weekend), the extraction vessels should be stored in a refrigerator.

Filter the slurry through a dense filter (5.4) into a calibrated flask (of capacity 50 ml or 100 ml) with a stopper. Wash the extraction vessel with ethanol (4.2) to remove residual extract and transfer quantitatively while rinsing the filter into the calibrated flask. Fill to the mark, stopper and mix thoroughly. This is the extract volume V_e.

Proceed with step 7.4.

7.3 Extraction variant B

Dispense an exact volume V_E (usually 20 ml or 25 ml) of the ethanol (4.2) into the extraction vessel (5.6) and allow the filter pieces to submerge. Close the screw cap tightly to avoid losses from evaporation of extractant. Shake slightly to resuspend the filter residue. Place the tube in the water bath (5.5) so that the extractant level aligns with the level of the bath. Heat for 5 min, shaking slightly if necessary. Take the extraction vessels from the water bath and allow to cool to room temperature for 15 min.

The time between extraction and measurement should be kept to a minimum.

NOTE 3 Extracts at this stage may be stored in a refrigerator overnight prior to measurement (see 7.4). Prolonged storage shall not exceed 3 d.

Filter the supernatant extract through a filter (5.4) into a clean extraction vessel (5.6) but do not rinse with fresh solvent (see note 4).

Alternatively, centrifuge the extraction vessels for a period which is sufficient to obtain a clear supernatant.

Use a clear extract or supernatant for photometry.

NOTE 4 Using this procedure, it is sufficient to recover merely a part of the extract volume, because the initial volume V_e of extractant is known exactly and is not reduced due to evaporation during extraction because the caps are tightly closed. Furthermore, the residual water content of the filters (see 7.1) is negligible, contributing to far less than 5 % of the extract volume.

7.4 Photometry

7.4.1 Transfer part of the clear extract into the spectrometer cuvette using a pipette, leaving a sufficient volume for the acidification step (see 7.4.2).

Measure the absorbance at 665 nm (A_{665}) and 750 nm (A_{750}) against a reference cell filled with ethanol (4.2).

NOTE 5 The absorbance at 665 nm should fall between 0,01 and 0,8 units. This may be achieved by suitably choosing the water volume filtered, extractant volume, dilution, optical pathlength, etc. To start with, take 0,5 litre of sample, a 50 mm diameter filter, 20 ml of ethanol and a 5 cm cuvette.

7.4.2 Acidify part of the extract (usually 5 ml to 10 ml) with 0,01 ml of hydrochloric acid (4.1) per 10 ml of extract volume, shake and measure the absorbance again at 665 nm and 750 nm, after 5 min to 30 min.

8 Calculation and expression of results

8.1 The chlorophyll-a concentration ρ_c, in micrograms per litre, is calculated according to the equation

$$\rho_c = \frac{(A - A_a)}{K_c} \times \frac{R}{R-1} \times \frac{10^3 V_e}{V_s \cdot d} \quad \ldots (1)$$

where

$A = A_{665} - A_{750}$	is the absorbance of the extract before acidification (see 7.4.1);
$A_a = A_{665} - A_{750}$	is the absorbance of the extract after acidification;
V_e	is the volume, in millilitres, of extract;
V_s	is the volume, in litres, of filtered sample;
$K_c = 82$ l/µg·cm	is the specific operational spectral absorption coefficient for chlorophyll-a (the value is taken from [2]);
$R = 1{,}7$	is the ratio A/A_a for a solution of pure chlorophyll-a which is transferred to phaeophytin by acidification (see 7.4.2) (the value is taken from [2]);
d	is the path length, in centimetres, of the optical cell;
10^3	is the dimension factor to fit V_e.

8.2 The phaeopigment concentration ρ_p, in micrograms per litre, is calculated according to the equation

$$\rho_p = A_a \times \frac{R}{K_c} \times \frac{10^3 V_e}{V_s \cdot d} - \rho_c \quad \ldots (2)$$

8.3 When the value 82 is taken for the specific spectral absorption coefficient for chlorophyll-a in 90 % ethanol, and 1,7 is taken for the maximum acid ratio (R) for pure chlorophyll-a, the chlorophyll-a concentration ρ_c in the water sample simplifies to

$$\rho_c = (A - A_a) \times 29{,}6 \times \frac{V_e}{V_s \cdot d} \quad \ldots (3)$$

and for the concentration of phaeopigment, equation 2 simplifies to equation (4)

$$\rho_p = A_a \times 20{,}8 \times \frac{V_e}{V_s \cdot d} - \rho_c \quad \ldots (4)$$

NOTES

6 The calculated values for phaeopigments are less reliable than for chlorophyll-a concentrations. Interlaboratory trials have shown an interlaboratory variation of 5 % to 11 % for chlorophyll-a determinations and 6 % to 46 % for phaeopigment determinations.

7 The ratio A/A_a is 1,7, if only undegraded chlorophyll-a is present in the sample. It is 1, if only degradation products of chlorophyll-a are present in the sample.

The operational absorption coefficient (82) for chlorophyll-a in ethanol (4.2) is derived from the operational absorption coefficient, recommended in [5] at 665 nm. The value (84) given there makes allowance for the presence of chlorophyll-b and chlorophyll-c. The absorbance of equal concentrations of chlorophyll-a in acetone is 2 % to 3 % higher than in ethanol at 665 nm [2] [4].