SLOVENSKI STANDARD
oSIST prEN 18034:2024

01-januar-2024

Alge in izdelki iz alg - Metode vzorčenja in analize - Določevanje vsebnosti klorofila

Algae and algae products - Methods of sampling and analysis - Determination of chlorophyll a content

Algen und algenbasierte Produkte oder Zwischenprodukte - Verfahren zur Probenentnahme und Analyse - Quantifizierung von Chlorophyll

Algues et produits ou intermédiaires à base d'algues - Méthodes d'échantillonnage et d'analyse - Détermination de la teneur en chlorophylle a

Ta slovenski standard je istoveten z: **prEN 18034**

ICS:

13.020.55	Biološki izdelki	Biobased products

oSIST prEN 18034:2024 **en,fr,de**

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

DRAFT
prEN 18034

November 2023

ICS

English Version

Algae and algae products or intermediates - Methods of sampling and analysis - Determination of chlorophyll *a* content

Algues et produits à base d'algues - Méthodes d'échantillonnage et d'analyse - Détermination de la teneur en chlorophylle *a*

Algen und Algenprodukte - Methoden zur Probeentnahme und Analyse - Bestimmung von Chlorophyll *a* gehalts

This draft European Standard is submitted to CEN members for enquiry. It has been drawn up by the Technical Committee CEN/TC 454.

If this draft becomes a European Standard, CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration.

This draft European Standard was established by CEN in three official versions (English, French, German). A version in any other language made by translation under the responsibility of a CEN member into its own language and notified to the CEN-CENELEC Management Centre has the same status as the official versions.

CEN members are the national standards bodies of Austria, Belgium, Bulgaria, Croatia, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Republic of North Macedonia, Romania, Serbia, Slovakia, Slovenia, Spain, Sweden, Switzerland, Türkiye and United Kingdom.

Recipients of this draft are invited to submit, with their comments, notification of any relevant patent rights of which they are aware and to provide supporting documentation.

Warning : This document is not a European Standard. It is distributed for review and comments. It is subject to change without notice and shall not be referred to as a European Standard.

EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

Contents

Page

European foreword

This document (prEN 18034:2023) has been prepared by Technical Committee CEN/TC 454 “Algae and algae products”, the secretariat of which is held by NEN.

This document is currently submitted to the CEN Enquiry.

This document has been prepared under a standardization request addressed to CEN by the European Commission.

Introduction[1]

This document has been prepared by the experts of CEN/TC 454 'Algae and algae products'.

The European Committee for Standardization (CEN) was requested by the European Commission (EC) to draft European standards or European standardization deliverables to support the implementation of Article 3 of Directive 2009/28/EC for algae and algae-based products or intermediates.

This request, presented as Mandate M/547[2], also contributes to the Communication on “Innovating for Sustainable Growth: A Bio economy for Europe”.

The former working group CEN Technical Board Working Group 218 “Algae”, was created in 2016 to develop a work programme as part of this Mandate. The technical committee CEN/TC 454 'Algae and algae products' was established to carry out the work programme that will prepare a series of standards.

The interest in algae and algae-based products or intermediates has increased significantly in Europe as a valuable source including but not limited to, carbohydrates, proteins, lipids, and several pigments. These materials are suitable for use in a wide range of applications from food and feed purposes to other sectors, such as textile, cosmetics, biopolymers, biofuel and fertilizer/biostimulants. Standardization was identified as having an important role in order to promote the use of algae and algae products.

The work of CEN/TC 454 should improve the reliability of the supply chain, thereby improving the confidence of industry and consumers in algae, which include macroalgae, microalgae, cyanobacteria, Labyrinthulomycetes, algae-based products or intermediates and will promote and support commercialisation of the European algae industry.

Given the importance of chlorophyll *a* in algae and algae products, it is important to have a good standardized method to determine the amount of chlorophyll *a*.

[1] Text in grey is fixed (based on CEN/TC 454 Decision 5-2019 taken by CEN/TC 454 on 2019-01-29 / Subject: CEN/TC 454 – Approval of the General Introduction Text).
[2] Available at http://ec.europa.eu/growth/tools-databases/mandates/index.cfm?fuseaction=refSearch.search#

1 Scope

This document specifies a laboratory method for the determination of chlorophyll *a* content in algae. The method was initially tested and evaluated on the microalgae species *Nannochloropsis* and a heat treated algal product tomato soup with *Nannochloropsis* supplement, and the macro algae species *Ulva* sp, *Furcellaria lumbricalis*, and *Saccharina latissima.* During an Interlaboratory Trial the method was tested on the microalgae species *Nannochloropsis* and the macro algae species *Saccharina latissima*. The microalgae species *Nannochloropsis* and *Phaodactylum* and the macro algae species *Ulva* sp and *Saccharina latissima* were tested in a Round Robin test. This document is only validated for chlorophyll *a*, but it can be used for other chlorophylls as well.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 17399, *Algae and algae products - Terms and definitions*

EN 17605:2022, *Algae and algae products - Methods of sampling and analysis - Sample treatment*

3 Terms and definitions

For the purpose of this document, the terms and definitions given in EN 17399 and the following apply.

3.1
chlorophylls
chlorophyll *a*, *b*, *c*1, *c*2 and *d* excluding degradation products

3.2
carotenoids
all accessory pigments of the class of tetraterpenoids including carotenes and xanthophylls

3.3
carotenes
unoxygenated (oxygen free) carotenoids such as α-carotene, β-carotene, and lycopene

3.4
chlorophyll standard
solution with defined concentration of the chlorophyll of interest that is used to determine the analyte of interest with the help of standard curves

Note 1 to entry: This is explained in further detail in Annex A.

3.5
degradation product
result of physical or chemical interaction by e.g. light, enzymatic, heat or acid treatment, leaving behind a fully or partially degraded pigment compound

3.6
mother solution
initial part of the standard solution, from which the dilutions for making standard curves are taken

3.7
pheophytin a
degradation product of chlorophyll *a*

3.8
pigments
all coloured substances in algae and algae products, such as chlorophylls, carotenoids and phycobiliproteins including their degradation products

3.9
xanthophylls
carotenoid molecules containing oxygen, such as lutein and zeaxanthin

4 Principle

The chlorophylls are extracted from the sample with methanol and separated using HPLC. The separated components are analysed at 440 nm using a photodiode array detector.

5 Apparatus

Usual laboratory apparatus and, in particular, the following:

5.1 Analytical balance, with an accuracy of preferably 0,01 mg.

5.2 High Performance Liquid Chromatograph (HPLC) with a Photodiode Array detector (PDA).

5.3 Centrifuge.

5.4 Vortex.

5.5 Spectrophotometer.

5.6 Fume hood.

5.7 Storage container, dark for storing the samples prior to analysis.

6 Reagents and materials

6.1 HPLC reagents.

All reagents should be HPLC grade.

For the triple gradient (see 8.6.1.1).

6.1.1 Mixture of methanol with a volume fraction of 80 % and 0,5M ammonium acetate buffer pH 7,2 with a volume fraction of 20 %.

6.1.2 Mixture of acetonitrile with a volume fraction of 90 %, and purified water, e.g. Milli-Q with a volume fraction of 10 %.

6.1.3 Ethyl acetate.

For the double gradient (see 8.6.1.2):

6.1.4 Mixture of methanol with a volume fraction of 70 %, and 0,028 M TBAA (tetrabutylammonium acetate) in purified water, e.g. Milli-Q.

6.1.5 Methanol.

6.2 HPLC column.

For the triple gradient (see 8.6.1.1).

6.2.1 C18 stationary phase, e.g. Nova-pak 4 µm (3,9 × 150 mm).

For the double gradient (see 8.6.1.2).

6.2.2 C8 stationary phase e.g. Zorbax Eclipse XDB-C8 3,5 µm (4,6 × 150 mm).

6.3 Pyrex screw capped tubes, 10 mL (1 / test portion) and 20 mL (1 / test portion).

6.4 Micropipettes.

6.5 Pasteur pipettes.

6.6 Glass beads, Ø 0,75 mm to 1 mm.

6.7 HPLC vials and caps.

6.8 Syringe, with volume of 1 mL and filters (0,20 µm, Ø 15 mm).

6.9 Methanol with a volume fraction of ⩾ 99,0 %.

6.10 Quartz cuvettes.

6.11 Chlorophyll standards, for the identification and quantification of chlorophyll, that shall comply to the following quality parameters:

— A concentration range from 0,5 to 3,0 µg/ml;

— Dissolved in solvent

— Transported on dry ice by the supplier

— Stored after arrival at least at −20 °C and preferably at −80 °C

Recommendations for chlorophyll standards are given in Annex A.

6.12 Aluminium foil.

7 Sampling and preparation of the sample

7.1 Sampling

It is important that the laboratory receives a sample that has not been damaged or changed during transport or storage.

Sampling is not part of the method specified in this document.

7.2 Sample treatment

Sampling and sample treatment are not parts of the method specified in this document. A recommended sample handling procedure is given in EN 17605:2022 with the following adaptations / additions:

— fine grinding shall be conducted as defined in 3.13 in EN 17605:2022;

— transport shall be performed on dry ice;

— storage shall be at −80°C. Also, macroalgae need to be freeze-dried.

Extensive storage times of the test sample should be avoided. In case this could not be avoided, the test sample should be freeze-dried again before usage.

Three test portions from the test sample shall be analysed as to be able to calculate the mean and standard deviation.

The samples shall be protected from light at any time before and during the analysis, by using e.g. aluminium foil.

Samples shall not be stored for over one month, because storage can degrade chlorophylls, even when the samples are frozen.

8 Procedure

8.1 Extraction

The whole extraction procedure, except for the weighing, shall be performed under a fume hood.

The extraction procedure shall be conducted through the following steps:

1) Weigh a test portion of approximately 25 mg of the test sample in a 10 mL pyrex screw capped tube (i.e. weight test portion);

2) Record the weight to the nearest 0,01 mg;

3) Add approximately 850 mg glass beads;

4) Add 5 mL methanol;

5) Vortex for 30 s;

6) Centrifuge at 450 g for 10 min;

7) Transfer the solvent to a 20 mL pyrex screw capped tube, e.g. by using a Pasteur pipette;

8) Leave the pellet in the 10 mL pyrex tube;

9) Repeat step 4) to 8) three more times, which should result in a pooled extract of 4 consecutive extractions;

10) Homogenize the pooled extract with a total volume of 20 mL by turning the tube upside down;

11) Transfer the extract to the HPLC vial with a syringe using a 0,20 µm filter.

The chlorophyll concentration decreases during the storage of extracts, even at temperatures of −20 °C and −80 °C. Therefore, extracts should be put on HPLC as soon as possible after the extraction.

8.2 Determination of the concentration of the chlorophyll *a* standard

For the calibration curve, the concentration of the chlorophyll *a* standard shall be measured spectrophotometrically as described below using published extinction coefficients found in Table 1.

Make a mother solution with a volume fraction of 90 % acetone, containing 0,025 mg/mL BHT, with a concentration of the standard of 1 µg/mL.

Keep solutions in fume hood and far away from direct light. Absorbance (Amax) is measured in a 1-cm cuvette at the standard's wavelength λmax (given with the published extinction coefficient) and at 750 nm to correct for light scattering. Note that the absorbance at known λmax of the chlorophylls should be in the range of 0.2-0.8. If the absorbance is outside this range then dilute the solution until the absorbance is within this range.

Also for newly purchased chlorophyll standards the concentration should be measured spectrophotometrically, because some degradation can already have taken place after the concentration determination by the supplier.

In order to determine the concentration of chlorophylls, the following formula shall be used:

$$CiSTD = 10^3 \frac{Ai(\lambda imax) - Ai(750)}{b\,\alpha} \quad [1]$$

where

CiSTD is the concentration (in µg/mL) of the standard for pigment i;

Ai(λimax) is the absorbance at λmax;

Ai(750) is the absorbance 750 nm;

b is the path length of the cuvette (cm) (= 1 cm); and

α is the weight-specific extinction coefficient (L g^{-1} • cm^{-1}) of chlorophyll i (see Table 1).

Table 1 Extinction coefficients of chlorophylls and degradation products based on Appendix G of Jeffrey et al. [1]

Name pigment	***Solvent***	Λ(nm)	α(L g^{-1} cm^{-1})
Chlorophyll *a* (MVChla)	90 % acetone	664.3	87.67
Chlorophyll *b*	90 % acetone	646.8	51.36
Chlorophyll *c2*	90 % acetone	443.8	374.00
Pheophorbide a	90 % acetone	667.0	74.20
Pheophytine a	90 % acetone	667.0	51.20

8.3 Preparation of the calibration curve

A calibration curve shall be prepared by diluting chlorophyll standards with the methanol extraction solvent . Multipoint calibrations shall always be performed. Therefore, at least 6 dilutions of the mother solution should be prepared, with a concentration range between 0,01 to 1,5 µg/mL. For every dilution, the peak area shall be determined twice by HPLC, using the method as described in 8.5, and the calibration curve shall be made as peak area versus concentration. A calibration curve shall be made based on the duplicate results for each concentration. The resulting calibration curves should be linear according to:

$$y = ax + b \quad [2]$$

where

y = the absorbance;

x = the concentration in µg/mL;

a = the slope;

b = y-intercept.

It should be noted that the determination coefficients (R^2 values) may never be less than 0,99.

A new calibration curve shall be constructed in the following cases:

- A new reference standard has been bought;
- A new type of chlorophyll has to be analysed;
- Another gradient is used;
- The type of stationary phase used, is changed;
- The settings of the HPLC method have been changed;
- The HPLC has been moved to another location.

8.4 Quality assurance and quality control equipment

Calibration curves should be checked regularly. At least, once a year new chlorophyll standards shall be purchased to make calibration curves.

When initiating a new sequence of samples on the HPLC device, 3 quality checks from the dilution series should be performed in order to ensure the quality assurance and quality control of the equipment. For these quality checks, 3 vials with known concentrations, which were previously run when creating calibration curves, should be used. It does not matter which concentration is chosen, as long as the concentration was initially measured when creating the standard curve, in order to enable the comparison of the resulting peak areas with the earlier obtained ones. The quality is considered good, when the deviation in the resulting concentrations is not over 10 %.

After measuring the chlorophyll standards for the first time, the cap on the vials shall be renewed. The vials shall be stored at a temperature of minimum −20 °C. If possible, storage at −80 °C is recommended.

8.5 HPLC analysis

8.5.1 HPLC setup

It is recommended to use the triple gradient (see 8.5.2) with the C18 stationary phase (see 6.2.1). In case your equipment is not suited for applying a triple gradient, the double gradient (see 8.5.3) may be used with the C8 stationary phase (see 6.2.2).

8.5.2 Triple gradient

8.5.2.1 Mobile phases

- A: Mixture of methanol with a volume fraction of 80 % and 0,5M ammonium acetate buffer pH 7,2 with a volume fraction of 20 %
- B: Mixture of acetonitrile with a volume fraction of 90 %, and purified water, e.g. Milli- Q with a volume fraction of 10 %
- C: Ethyl acetate