SLOVENSKI STANDARD
oSIST prEN 17908:2022

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Alge in izdelki iz alg - Metode vzorčenja in analize - Določevanje skupnih lipidov z metodo po Ryckebosch-Foubertu

Algae and algae products - Methods of sampling and analysis - Determination of total lipids content using the Ryckebosch-Foubert method

Algen und algenbasierte Produkte oder Zwischenprodukte - Methoden zur Probeentnahme und Analyse - Bestimmung von Gesamtlipiden mit der Ryckebosch-Foubert Methode

Algues et produits à base d'algues - Méthodes d'échantillonnage et d'analyse - Détermination de la teneur en lipides totaux à l'aide de la méthode de Ryckebosch-Foubert

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Algae and algae products - Methods of sampling and analysis - Determination of total lipids content using the Ryckebosch-Foubert method

Algues et produits à base d'algues - Méthodes d'échantillonnage et d'analyse - Détermination de la teneur en lipides totaux à l'aide de la méthode de Ryckebosch-Foubert

Algen und algenbasierte Produkte oder Zwischenprodukte - Methoden zur Probeentnahme und Analyse - Bestimmung von Gesamtlipiden mit der Ryckebosch-Foubert Methode

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.

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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.

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

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European foreword

This document (prEN 17908:2022) has been prepared by Technical Committee CEN/TC 454 “Algae an 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 mandate given to CEN by the European Commission and the European Free Trade Association.

Introduction

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[1], 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 textiles, cosmetics, biopolymers, biofuel and fertilizer/biostimulants. Standardization was identified as having an important role in promoting 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 commercialization of the European algae industry.

This document has been developed with the aim to cover the horizontal definitions for algae and algae-based products or intermediates. Hence, other terms and definitions are given in the other standards developed by CEN/TC 454 “Algae and algae products”.

For food, feed and non-food, non-feed applications additional definitions can exist in other product specific standards.

[1] 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 the total lipid content in micro- and macroalgae by the Ryckebosch-Foubert method.

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 17399:2020, *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 purposes of this document, the terms and definitions given in EN 17399:2020 and the following apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

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

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

3.1
total lipid content
all lipid substances extracted from the test portion under the operating conditions specified, expressed in % or ‰ (i.e. mg/gram or g/kg) relative to dry weight

4 Principle

The lipids in dried and homogenized algal biomass are extracted with chloroform/methanol. Afterwards a counter extraction with water is performed to remove the non-lipids. Two consecutive extractions of two phases each are performed, as has been optimized as described in Annex A. After removal of the solvents, the amount of total lipids is determined gravimetrically.

5 Apparatus

5.1 Rotary evaporator, including a vacuum pomp with an adjustable vacuum level

5.2 Centrifuge, at room temperature

5.3 Vortex

5.4 Analytical balance, with a readability of preferably 0,01 mg

5.5 Bead beater (only in case procedure 8.2 is used)

Different bead beaters and / or different cells can lead to different results. Therefore, when using procedure 8.2, the specifications of the bead beater and cell used shall be indicated in the report.

6 Reagents and materials

6.1 Pyrex screw capped tubes, 10 ml (1/test portion) or comparable glassware

6.2 Pyrex screw capped tubes, 20 ml (1/test portion) or comparable glassware

6.3 Funnels, (1/test portion)

6.4 Filter paper of 180 µm, (1/test portion), e.g. of Whatman, type nr. 1

6.5 Pasteur pipettes

6.6 Round-bottom flasks, useable for analytical balance (not above the maximum weight) for the rotary evaporator system (1/test portion), at the temperature of the balance (to avoid weighing error)

6.7 Micropipettes

6.8 Chloroform (with a purity not less than 99,0 % (V/V)

6.9 Methanol (with a purity not less than 99,0 % (V/V)

6.10 Chloroform/methanol (1:1 by volume)

6.11 Anhydrous sodium sulphate

6.12 Demineralized water

7 Sampling and sample handling

Sampling is not part of the method specified in this document. A recommended sampling 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, macro-algae 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.

8 Procedure

8.1 General

Some algae, cultivated under specific culture conditions, can have a high lipid content and/or a rigid cell envelope that makes that cell disruption is required prior to the extraction. Procedure 8.2 has been established for these cases. For a species or culture condition that has not been analysed before, both procedures 8.1 and 8.2 shall be performed and the results compared. In this case, first perform both procedure (8.1 without cell disruption and 8.2 with cell disruption) and compare the results. In case the total lipid content using procedure 8.2 is significantly (Level of significance: 0,01) higher than that using procedure 8.1, continue to use procedure 8.2 for that sample, otherwise stick to 8.1.

It is not appropriate to always incorporate this cell disruption step as for most samples it is not necessary and even leads to lower values (due to loss on beads) and more variability in the results. It also implies that special instrumentation should be available in the lab.

For all standard algae, cultivated under standard cultivation conditions, no cell disruption step is necessary to extract all the lipids and procedure 8.1 shall be used.

8.2 Procedure without cell disruption

8.2.1 General

The procedure without cell disruption shall be conducted through the following steps. The complete procedure, except for the weighing, should be conducted under the fumehood.

8.2.2 First extraction

a) Weigh a test portion of approximately 100 mg of the test sample in a 10 ml pyrex screw capped tube (i.e. weight test portion). Record the weight to the nearest 0,01 mg (or 0,1 mg depending on the readability of your analytical balance);

b) Add 4 ml of methanol followed by 2 ml of chloroform;

c) Add 0,4 ml demineralized water;

d) Vortex at least 30 s until the different layers are mixed well;

e) Add 2 ml of chloroform followed by 2 ml of water (10 ml pyrex tube is now totally filled);

f) Vortex at least 30 s until the different layers are mixed well;

g) Centrifuge the tube at 450 g for 10 min;

h) After centrifugation 2 solvent phases are formed (often separated by the biomass pellet): remove the upper layer with a Pasteur pipette to a waste container;

NOTE This layer is waste and contains sugars, proteins and minerals.

i) Transfer the lower layer to a 20 ml pyrex screw capped tube using a Pasteur pipette. For each test portion another Pasteur pipette shall be used. This pipette should be kept in the 20 ml tube for the second extraction;

   It is recommended to tilt the tube about 45°. By doing this, the pellet will move and some space will be available to move the Pasteur pipette along the biomass pellet without touching it. It is possible that the pellet breaks in smaller parts during tilting, which is not a problem.

j) Leave the pellet in the 10 ml pyrex tube;

k) Add 4 ml of chloroform/methanol (1:1) to this pellet;

l) Vortex at least 30 s until the different layers are mixed well;

m) Centrifuge the tube at 450 g for 10 min;

n) After centrifugation, only 1 phase is observed. Transfer this solvent layer to the same 20 ml pyrex tube as before with the same Pasteur pipette as before.

8.2.3 Second extraction

Repeat step a) from the first extraction on the same remaining biomass pellet. (Do not weigh new biomass). Then continue with the following steps:

a) Add 4 ml of methanol followed by 2 ml of chloroform;

b) Vortex at least 30 s until the different layers are mixed well;

c) Add 2 ml of chloroform followed by 2 ml of water (10 ml pyrex tube is now totally filled);

d) Vortex at least 30 s until the different layers are mixed well;

e) Centrifuge the tube at 450 g for 10 min;

f) After centrifugation 2 solvent phases are formed: remove the upper layer with a Pasteur pipette to a waste container;

g) Transfer the lower layer to the same 20 ml pyrex tube as before with the same Pasteur pipette that is still in the tube. After this step, the pipette can be thrown away;

h) Leave the pellet in the 10 ml pyrex tube;

i) Add 4 ml of chloroform/methanol (1:1) to this pellet;

j) Vortex at least 30 s until the different layers are mixed well;

k) Weigh the round-bottom flask (previously dried at 103°C in the oven and then cooled) on the analytical balance to the nearest 0,01 mg (i.e. weight empty flask) (or 0,1 mg depending on the readability of your analytical balance);

l) Equip the round-bottom flask with a funnel and filter paper nr. 1. Add 5 g of sodium sulphate in the filter paper;

It is very important that no sodium sulphate powder comes in the flask, otherwise the total lipid content will be overestimated. The presence of crystals after drying is an indication that sodium sulphate has been spilled into the flask. If this occurs, the method shall be started over with a new sample. Extra caution is recommended.

m) Filter the solvent from the 20 ml pyrex tube through the filter paper with sodium sulphate layer to remove remaining water;

n) Do the same with the solvent from the 10 ml pyrex tube which contains only 1 solvent layer after re-extraction and the pellet. When filtering the solvent through the filter paper, the pellet can also end up in the filter paper. This is not a problem;

o) After filtering, when the 10 and 20 ml tubes are empty, the tubes and filter paper are rinsed carefully with chloroform / methanol (1:1). Tubes are rinsed twice, each time using the volume of one Pasteur pipette. Filter papers are rinsed three times, each time with 6 times the volume of one Pasteur pipette (some green haze can remain on the filter paper). The amount of chloroform /methanol need not be strictly accurate;

p) When all the solvent is in the flask, it can be removed with a rotary evaporator system under reduced pressure with the following program:

   — from 300 mbar to 270 mbar over 2 min period;

   — 270 mbar to 160 mbar over 5 min period;

   — hold for 5 min at 160 mbar;

   — 160 mbar to 43 mbar over 1 min period;

   — hold for 4 min at 43 mbar.

   During the process, the round-bottom flask gently (100 – 200 rpm) rotates in a warm water bath at 40°C (not higher). The last bits of condensing water shall be removed by flushing manually with nitrogen. In order to do so, use a connection tubing with Pasteur pipette on the nitrogen source and move with the tip of the Pasteur pipette in the flasks. If flushing with nitrogen is not possible, simply omit this step (or keep on the rotavapor longer or leave overnight in the fumehood) and do not replace by heating at temperatures higher than 40°C.

q) Allow the flask to cool to room temperature in a desiccator for 15 min and weigh again to the nearest 0,01 mg (i.e. weight flask after extraction) (or 0,1 mg depending on the readability of your analytical balance).

8.3 Procedure with cell disruption

8.3.1 General

The procedure with cell disruption shall be conducted through the following steps. The complete procedure, except for the weighing, shall be conducted under the fumehood.

8.3.2 First extraction

a) Weigh a test portion of approximately 100 mg of the test sample in the recipient of the bead beater (i.e. weight test portion);

b) Record the weight to the nearest 0,01 mg (or 0,1 mg depending on the readability of your analytical balance);

c) Add 2 ml of methanol and one bead;

d) Bead beat for 15 min at 30 Hz;

e) Transfer the solvent to a 10 ml pyrex tube;

f) Add 2 ml of methanol to the recipient of the bead beater, shake and transfer the solvent to the 10 ml pyrex tube;

g) Add 2 ml of chloroform to the recipient of the bead beater, shake and transfer the solvent to the 10 ml pyrex tube;

h) Add 0,4 ml demineralized water to the 10 ml pyrex tube;

i) Vortex at least 30 s until the different layers are mixed well;