BRITISH STANDARD

BS EN ISO 29841:2014 +A1:2016

Vegetable fats and oils — Determination of the degradation products of chlorophylls a and a' (pheophytins a, a' and pyropheophytins)

ICS 67.200.10

BSI British Standards

National foreword

This British Standard is the UK implementation of EN ISO 29841:2014+A1:2016. It is identical to ISO 29841:2009, incorporating amendment 1:2016. It supersedes BS EN ISO 29841:2014, which is withdrawn.

The start and finish of text introduced or altered by amendment is indicated in the text by tags. Tags indicating changes to ISO text carry the number of the ISO amendment. For example, text altered by ISO amendment 1 is indicated by [A1] [A1].

The UK participation in its preparation was entrusted to Technical Committee AW/307, Oilseeds, animal and vegetable fats and oils and their by-products.

A list of organizations represented on this committee can be obtained on request to its secretary.

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

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30 April 2014	This corrigendum renumbers BS ISO 29841:2009 as BS EN ISO 29841:2014
31 March 2016	Implementation of ISO amendment 1:2016 with CEN endorsement A1:2016

EUROPEAN STANDARD

NORME EUROPÉENNE

EUROPÄISCHE NORM

EN ISO 29841:2014+A1

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ICS 67.200.10

English Version

Vegetable fats and oils - Determination of the degradation products of chlorophylls a and a' (pheophytins a, a' and pyropheophytins) (ISO 29841:2009)

Corps gras d'origine végétale - Détermination des produits de décomposition des chlorophylles a et a' (phéophytines a, a' et pyrophéophytines) (ISO 29841:2009)

Pflanzliche Fette und Öle - Bestimmung thermischer Abbauprodukte des Chlorophyll a und a' (Pheophytin a, a' und Pyropheophytin) (ISO 29841:2009)

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Foreword

The text of ISO 29841:2009 has been prepared by Technical Committee ISO/TC 34 “Food products” of the International Organization for Standardization (ISO) and has been taken over as EN ISO 29841:2014 by Technical Committee CEN/TC 307 “Oilseeds, vegetable and animal fats and oils and their by-products - Methods of sampling and analysis” the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by October 2014, and conflicting national standards shall be withdrawn at the latest by October 2014.

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Endorsement notice

The text of ISO 29841:2009 has been approved by CEN as EN ISO 29841:2014 without any modification.

Foreword to amendment A1

This document (EN ISO 29841:2014/A1:2016) has been prepared by Technical Committee ISO/TC 34 “Food products” in collaboration with Technical Committee CEN/TC 307 “Oilseeds, vegetable and animal fats and oils and their by-products - Methods of sampling and analysis” the secretariat of which is held by AFNOR.

This Amendment to the European Standard EN ISO 29841:2014 shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by August 2016, and conflicting national standards shall be withdrawn at the latest by August 2016.

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Endorsement notice

The text of ISO 29841:2009/Amd 1:2016 has been approved by CEN as EN ISO 29841:2014/A1:2016 without any modification.

Vegetable fats and oils — Determination of the degradation products of chlorophylls *a* and *a'* (pheophytins *a, a'* and pyropheophytins)

1 Scope

This International Standard specifies a procedure for the determination of the degradation products pheophytin *a*, *a'* and pyropheophytin *a* of chlorophylls. The method is applicable to vegetable fats and oils only.

2 Normative references

The following referenced documents are indispensable for the application 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.

ISO 661, *Animal and vegetable fats and oils — Preparation of test sample*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
pyropheophytin *a* (content in chlorophyll degradation products)
mass of pyropheophytin *a* divided by the sum of the masses of pheophytin *a*, *a'* and pyropheophytin *a*

4 Principle

Separation of the pigments (chlorophylls, pheophytins *a* and *a'*, pyropheophytin *a*) using a miniaturized column chromatography on a silica gel column from the major part of the lipids (triglycerides). The eluate is analysed by RP18-HPLC, and the separated components are monitored at 410 nm using a photometric detector.

The analysis of some seed oils (e.g. rapeseed oil) requires a higher sensitivity which can be achieved using fluorescence detection with an excitation wavelength of 430 nm and an emission wavelength of 670 nm.

A direct HPLC determination with fluorescence detection is also possible and described in Annex C.

5 Reagents

WARNING — Attention is drawn to the regulations which specify the handling of hazardous substances. Technical, organizational and personal safety measures shall be followed.

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade.

5.1 Chlorophyll *a*.

NOTE Due to the lack of pheophytin and pyropheophytin reference materials, chlorophyll *a* is used to produce these degradation products. Pheophytins are obtained from a chlorophyll *a* solution by acidification; pyropheophytins by additional heat (> 100 °C) (Reference [4]).

5.2 Acetone.

5.3 Methanol.

5.4 Diethyl ether.

5.5 Petroleum ether, boiling point range 40 °C to 60 °C.

5.6 *n*-Heptane or, if not available, ***n*-hexane**.

CAUTION — Owing to its toxicity, avoid using *n*-hexane if *n*-heptane is available.

5.7 Petroleum ether and diethyl ether solvent mixture, volume fractions: $\varphi_{PetE} = 90$ ml/100 ml; $\varphi_{Et_2O} = 10$ ml/100 ml.

6 Apparatus

Usual laboratory equipment, and in particular the following.

6.1 HPLC setup.

6.1.1 HPLC system, consisting of a pump, a sample-injecting device (20 µl loop) or auto sampler, either a photometric detector for measurements at 410 nm or a fluorescence detector (excitation wavelength, $\lambda_{ex} = 430$ nm and emission wavelength, $\lambda_{em} = 670$ nm), and an integration system.

6.1.2 HPLC column, 250 mm length, 4,0 mm or 4,6 mm internal diameter, filled with reverse-phase type RP18 particles, size 5 µm.

The following conditions have been found to be suitable:

Stationary phase: Partisil ODS 3, 5 µm, 250 mm × 4,6 mm [1)];

Mobile phase: Water, methanol, and acetone (6.1.3);

Flow rate: 1,0 ml/min.

6.1.3 Mobile phase, water, methanol, and acetone mixture, volume fractions: $\varphi_{H_2O} = 4$ ml/100 ml; $\varphi_{MeOH} = 36$ ml/100 ml; $\varphi_{Me_2C=O} = 60$ ml/100 ml. All solvents shall be HPLC grade.

6.2 Taper-shaped flask, 〚A1〛 of capacity 25 ml or 50 ml 〚A1〛.

6.3 Beakers, of different capacities.

6.4 Rotary evaporator, with water bath.

6.5 Auto sampler vials, of suitable capacity.

1) Example, available commercially, of a C18 reverse phase with a 10,5 % mass fraction carbon load, end-capped for deactivation of silanols to minimize the need for ion suppression or ion pairing agents. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

6.6 Silica cartridge, [A1] 500 mg/6 ml or 1 000 mg/6 ml, 55 µm, 7 nm or **diol cartridge**, 3 ml.

NOTE Also, in-house silica mini-columns can be used for the separation. For this, use Silica gel 60, for column chromatography (0,063 mm to 0,100 mm), adjusted to a moisture content of w = 5 % (mass fraction). Activate the silica gel by heating overnight at (160 ± 5) °C. After heating, place the silica gel in a desiccator for cooling and then transfer the silica gel to a stoppered flask. Add 5 % of water and shake until no lumps can be seen and the powder flows freely (1 h in an automatic shaking machine). Store the conditioned silica gel overnight before use.[2)] [A1]

7 Sample

7.1 Sampling

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 5555 [1].

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport or storage.

7.2 Preparation of the test sample

Prepare the test sample in accordance with ISO 661.

IMPORTANT — Test samples shall be stored cold and protected from light in glass vessels. After clean-up, the test solutions shall be analysed *immediately* to prevent degradation and oxidation. Avoid contact of test samples with plastics.

8 Procedure

8.1 Weigh about 300 mg of the test sample (7.2) into a small beaker (6.3), dissolve it in 1 ml of *n*-heptane or *n*-hexane (5.6) and pour the solution on to the silica cartridge (silica gel column) (6.6). Rinse the beaker twice with 1 ml portions of petroleum ether (5.5), pouring these washings on to the cartridge (column) also.

8.2 As soon as the solvent has drained to the top of the cartridge (column), elute the non-polar substances twice with 5 ml petroleum ether/diethyl ether solvent mixture (5.7) each time.

8.3 Elute the pheophytin fraction twice with 5 ml acetone (5.2) and collect this fraction in a taper-shaped flask (6.2) protected from light.

8.4 Evaporate the solvent to dryness on a rotary evaporator (6.4) at max 20 °C. Dissolve the residue in 200 µl acetone. Introduce this solution immediately into the HPLC setup (6.1).

IMPORTANT — Pheophytins are very unstable in light and oxidize easily.

8.5 Typical chromatograms run under the column conditions listed in 6.1.2 are shown in Figures A.1 and A.2.

2) Strata Si-1 (silica cartridge) and Supelclean LC-Diol SPE Tubes – Supelco 5 7016 (diol cartridge) are examples of suitable products available commercially. This information is given for the convenience of users of this [A1] document [A1] and does not constitute an endorsement by ISO of these products.

9 Results of the determination

9.1 Peak identification

The chromatographic pattern of the determination may show the peaks of the pigments chlorophyll *a*, chlorophyll *b*, the corresponding pheophytins and pyropheophytins. Reference should be made to the typical chromatograms presented in Figures A.1 and A.2 as the other pigments are not available as reference materials due to their instability. To identify pheophytin *a*, pheophytin *a′* and pyropheophytin *a*, see Figures A.1 and A.2.

9.2 [A1] Calculation of the relative pyropheophytin *a* content [A1]

Use the peak areas to calculate the relative proportions of the analytes in the sample solution. For the purpose of this method, it is assumed that the response factors of all pigments are equal. The pyropheophytin *a* content as a percentage mass fraction, w_{PPPa}, is calculated on the basis of a peak area ratio as follows:

$$w_{PPPa} = \frac{A_{PPPa} \times 100}{(A_{PPPa} + \sum A_{PP})}$$

where

A_{PPPa} is the peak area of pyropheophytin *a*;

ΣA_{PP} is the sum of the peak areas of pheophytin *a* and *a′*.

Report the result to one decimal place.

10 Precision of the method

10.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in Annex B. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

10.2 Repeatability

The absolute difference between two independent single test results, obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within a short interval of time, will in not more than 5 % of cases exceed the value of r given in Table B.1.

10.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5 % of cases exceed the value of R given in Table B.1.

11 Test report

The test report shall contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) the sampling method used, if known;

c) the test method used, with reference to this International Standard;

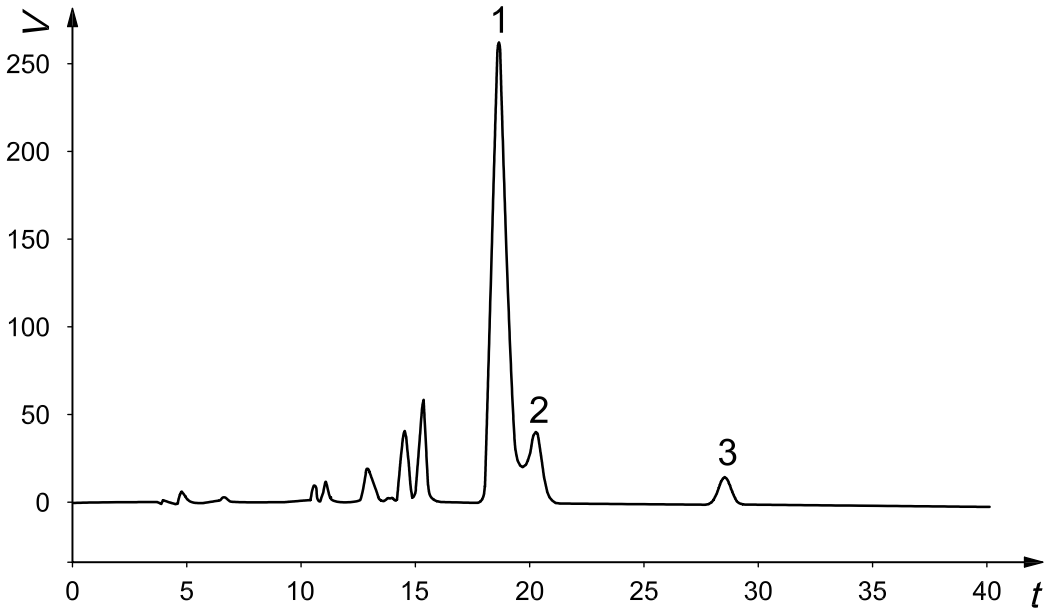
d) all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents that may have occurred when performing the method, which may have influenced the test result(s);

e) the test result obtained;

f) if the repeatability has been checked, the final quoted result obtained.

Annex A
(informative)

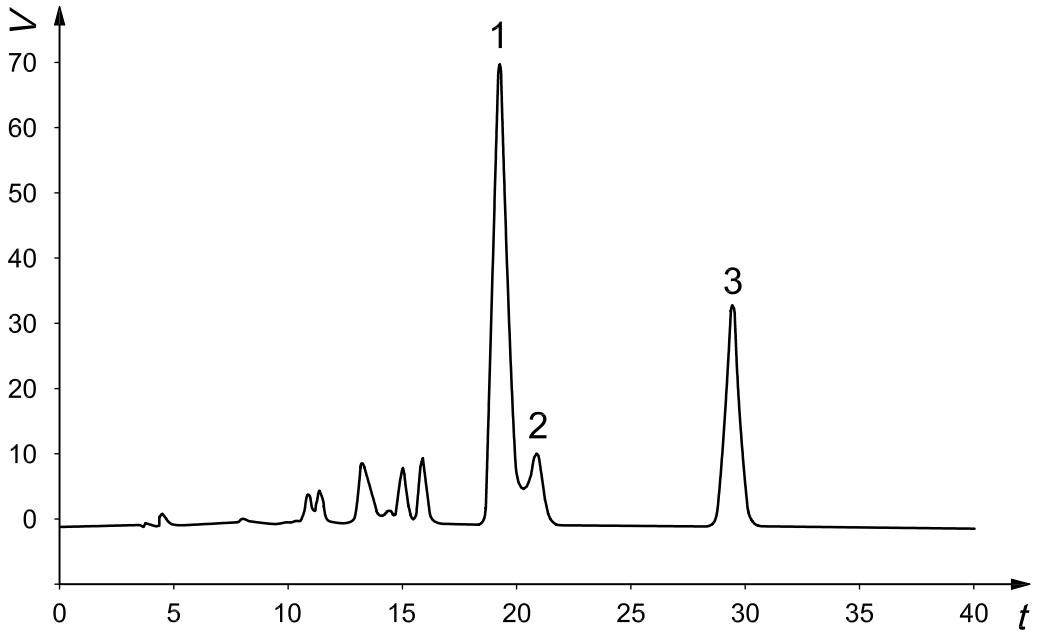
Example of a typical chromatogram



Key

1 pheophytin *a*
2 pheophytin *a'*
3 pyropheophytin
t elution time, min
V potential difference, volts

Figure A.1 — HPLC chromatogram of an extra virgin olive oil with 5 % mass fraction pyropheophytin content in chlorophyll degradation products



Key

1 pheophytin *a*
2 pheophytin *a*′
3 pyropheophytin
t elution time, min
V potential difference, volts

Figure A.2 — HPLC chromatogram of an extra virgin olive oil with 30 % mass fraction pyropheophytin content in chlorophyll degradation products

Annex B
(informative)

Results of an interlaboratory test

The precision of the method is the result of an interlaboratory study organized by the Joint Committee for the Analysis of Fats, Oils, Fat Products, Related Products and Raw Materials (DIN/DGF) in which 16 laboratories from France, Germany, and Italy participated. The study was carried out in 2005 on five samples, and precision data were calculated in accordance with ISO 5725-1 [2] and ISO 5725-2 [3].

Table B.1 — Summary of statistical results of olive oils

Sample	A	B	C	D	E
Number of participating laboratories, N	16	16	16	16	16
Number of laboratories retained after eliminating outliers, n	12	12	14	15	15
Number of individual test results of all laboratories on each sample, $\sum_{i=1}^{n} n_{w,i}$	24	24	28	30	30
Mean value, $\overline{w}_{PPPa}$, %	**28,86**	**34,01**	**5,63**	**6,26**	**84,75**
Repeatability standard deviation, s_r, %	0,53	0,61	0,59	0,36	1,09
Repeatability coefficient of variation, CV(r), %	1,8	1,8	10,4	5,8	1,3
Repeatability limit, r ($s_r \times 2{,}8$), %	**1,48**	**1,71**	**1,65**	**1,01**	**3,05**
Reproducibility standard deviation, s_R, %	1,78	2,08	0,79	1,29	3,06
Reproducibility coefficient of variation, CV(R), %	6,2	6,1	14,1	20,7	3,6
Reproducibility limit, R ($s_R \times 2{,}8$), %	**4,98**	**5,82**	**2,21**	**3,61**	**8,56**

Annex C
(informative)

Direct HPLC method with fluorescence detection

C.1 Principle

This annex describes an alternative method. The oils are submitted to HPLC without any prior treatment.

C.2 Solvents for HPLC

C.2.1 **Water**, for HPLC.

C.2.2 **Methanol**, for HPLC.

C.2.3 **Acetone**, for HPLC.

C.3 Apparatus

C.3.1 **Binary HPLC system**, consisting of a pump, a sample-injecting device (20 µl loop) or auto sampler, a fluorescence detector (λ_{ex} = 430 nm and λ_{em} = 670 nm), and an integration system.

C.3.2 **HPLC column**, 250 mm length, 4,0 mm or 4,6 mm internal diameter, filled with reverse-phase type RP18 particles, size 5 µm.

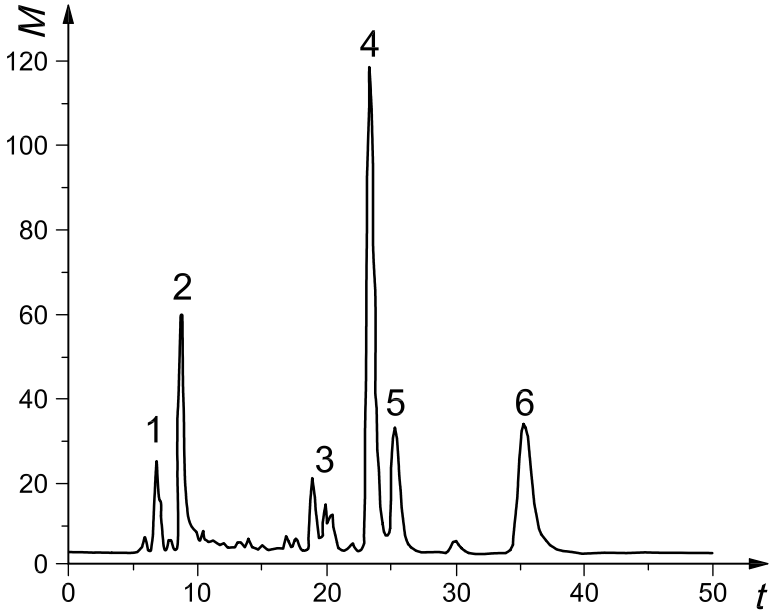
C.4 Procedure

C.4.1 Transfer the oils into an amber HPLC vial.

C.4.2 Introduce the test portion into the HPLC apparatus (Clause C.3). The conditions listed in Table C.1 have been found to be suitable and to give a typical chromatogram, such as that shown in Figure C.1.

Table C.1 — Elution programme with 1,0 ml/min

Time (min)	**Solvent A** Volume fractions: φ_{H_2O} = 4 %; φ_{MeOH} = 76 %; $\varphi_{Me_2C=O}$ = 20 %	**Solvent B** Volume fractions: φ_{H_2O} = 4 %; φ_{MeOH} = 36 %; $\varphi_{Me_2C=O}$ = 60 %
0	100 %	0 %
7	0 %	100 %
50	0 %	100 %
50,1	100 %	0 %
60	100 %	0 %



Key

1 pheophorbide
2 methylpheophorbide
3 pheophytin *b*
4 pheophytin *a*
5 pheophytin *a'*
6 pyropheophytin

M fluorescence detection, lux
t elution time, min

Figure C.1 — HPLC chromatogram of a rapeseed oil with pyropheophytin

Bibliography

[1] ISO 5555, *Animal and vegetable fats and oils — Sampling*

[2] ISO 5725-1, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[3] ISO 5725-2, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[4] AITZETMÜLLER, K. Chlorophyll-Abbauprodukte in pflanzlichen Ölen [Chlorophyll degradation products in vegetable oils]. *Fat Sci. Technol*. 1989, **91**, pp. 99-105; AITZETMÜLLER, K. Analysis of chlorophyll degradation products in fats and oilseeds. In: *Actes du congrès international «Chevreul» pour l'étude des corps gras* [Proceedings of the Chevreul International Congress], Angers, 1989-06-06/10, p. 177-84. Association française pour l'Étude des Corps gras, Neuilly sur Seine

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