BRITISH STANDARD

BS EN ISO
15302:2007

Animal and vegetable fats and oils — Determination of benzo[a]pyrene content — Reverse-phase high-performance liquid chromatography method

The European Standard EN ISO 15302:2007 has the status of a British Standard

ICS 67.200.10

National foreword

This British Standard is the UK implementation of EN ISO 15302:2007. It is identical to ISO 15302:1998. It supersedes BS 684-2.47:1998 which is withdrawn.

The UK participation in its preparation was entrusted to Technical Committee AW/307, Oil seeds, 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.

Compliance with a British Standard cannot confer immunity from legal obligations.

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Animal and vegetable fats and oils - Determination of benzo[a]pyrene content - Reverse-phase high-performance liquid chromatography method (ISO 15302:1998)

Corps gras d'origines animale et végétale - Détermination de la teneur en benzo[a]pyrène - Méthode par chromatographie liquide à haute performance à polarité de phase inversée (ISO 15302:1998)

Tierische und pflanzliche Fette und Öle - Bestimmung des Benzo[a]pyren-Gehalts - Umkehrphasen-HPLC-Verfahren (ISO 15302:1998)

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Foreword

The text of ISO 15302:1998 has been prepared by Technical Committee ISO/TC 34 "Agricultural food products" of the International Organization for Standardization (ISO) and has been taken over as EN ISO 15302:2007 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of an identical text or by endorsement, at the latest by July 2007, and conflicting national standards shall be withdrawn at the latest by July 2007.

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Endorsement notice

The text of ISO 15302:1998 has been approved by CEN as EN ISO 15302:2007 without any modifications.

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 15302 was prepared by Technical Committee ISO/TC 34, Agricultural food products, Subcommittee SC 11, Animal and vegetable fats and oils.

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

Animal and vegetable fats and oils — Determination of benzo[a]pyrene content — Reverse-phase high-performance liquid chromatography method

1 Scope

This International Standard specifies a method for the determination of benzo[a]pyrene in crude or refined edible oils and fats by reverse-phase high-performance liquid chromatography (HPLC) using fluorimetric detection in the range from 0,1 µg/kg to 10 µg/kg.

2 Normative reference

The following standard contains provisions, which through reference in this text, constitute provisions of this International Standard. At the time of publication the edition indicated was 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 edition of the standard indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 661:1989, Animal and vegetable fats and oils — Preparation of test sample

3 Principle

Adsorption of a suitable amount of sample on an alumina column, followed by elution with light petroleum of any benzo[a]pyrene present, and subsequent analysis of the eluate by HPLC using a fluorimetric detector.

4 Reagents

All reagents shall be of recognized analytical grade. Where analytical grade solvents other than the recommended ones are used, a full blank analysis shall be carried out and the results of this blank analysis reported.

4.1 Water, double distilled, filtered through a membrane filter of 0,45 µm pore size; deionized water obtained by purifying demineralized water systems may also be used.

4.2 Light petroleum (boiling point range between 40 °C and 60 °C), or **hexane**, redistilled over potassium hydroxide pellets (4 g/l).

4.3 Acetonitrile, suitable for HPLC.

4.4 Tetrahydrofuran, suitable for HPLC.

4.5 Toluene, suitable for HPLC.

4.6 Sodium sulfate, granular, anhydrous.

4.7 Alumina, activity grade 4, prepared from neutral aluminium oxide, activity grade super 1, deactivated by the addition of 10 ml distilled water to 90 g of alumina.

CAUTION — THE REACTION IS EXOTHERMIC AND PRESSURE MAY BUILD UP.

Shake the container for about 15 min and allow the contents to equilibriate for 24 h. Store the alumina in a closed vessel at ambient temperature.

4.8 Benzo[a]pyrene, 99,0 % purity.

CAUTION — BENZO[a]PYRENE IS A KNOWN CARCINOGEN. CARRY OUT ALL WORK WITH IT IN A FUME HOOD, WEARING GLOVES TO MINIMIZE EXPOSURE.

4.9 Benzo[a]pyrene solutions1)

4.9.1 Stock solution

Weigh, to the nearest 0,1 mg, about 12,5 mg of benzo[a]pyrene in a 25 ml graduated flask. Dissolve it in toluene (4.5) and fill to the mark.

This solution contains about 0,5 mg/ml benzo[a]pyrene and should be stored in the dark at 4 °C when it is stable for 6 months at least.

4.9.2 Standard solutions

Prepare two standard solutions containing approximately 0,2 µg/ml and 0,01 µg/ml of benzo[a]pyrene respectively by diluting aliquots of the stock solution (4.9.1) with acetonitrile (4.3).

5 Apparatus

Usual laboratory apparatus and, in particular, the following.

5.1 Glass column for chromatography, 300 mm long, 15 mm internal diameter, fitted with sintered glass discs, and polytetrafluoroethylene (PTFE) tap.

5.2 Water baths (two), maintained at 35 °C ± 1°C and 65 °C ± 1°C.

5.3 Flash evaporator

A rotary evaporator with vacuum and a water bath set at 40 °C may be used. Care should be taken to prevent cross contamination. Clean the system thoroughly between determinations.

5.4 High-performance liquid chromatograph, consisting of an HPLC pump, injection valve with 10 µl sample loop, reverse-phase column, electronic integrator and chart recorder.

NOTE If an autosampler is used, the sample loop should be flushed with acetonitrile between subsequent injections.

1) A suitable reference material is available from the Commission of the European Community Bureau of Reference (BCR), rue de la Loi 200, B-1049, Brussels, Belgium.

5.5 Columns for HPLC analysis

5.5.1 Reverse-phase guard column, capable of resolving benzo[a]pyrene from co-extractives, together with appropriate precolumn [e.g. stainless-steel precolumn 75 mm long, 4,6 mm internal diameter, packed with Lichrosorb RP-18 (5 µm particle size).2)

5.5.2 HPLC reverse-phase column, 250 mm long, of 4,6 mm internal diameter (stainless steel), for polycyclic aromatic hydrocarbons (PAHs) (e.g. Chromspher 5 PAH or Vydac 201 TP5).3)

5.6 Fluorescence detector, with emission wavelength at 406 nm (slit 10 nm) and excitation wavelength at 384 nm (slit 10 nm). The detector shall be capable of the required performance to carry out the analysis.

5.7 Crimp-top minivials, of about 1 ml volume, with Teflon-layered septa and aluminium caps.

5.8 Hand crimper, for crimping the caps onto the vials.

5.9 Disposable pipettes

6 Sampling

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

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

7 Preparation of test sample

Prepare the test sample in accordance with ISO 661.

8 Procedure

8.1 Clean up of sample

8.1.1 Weigh, to the nearest 0,001 g, about 2 g of the oil sample into a 10 ml graduated flask. Dissolve in light petroleum (4.2) and dilute to the mark.

8.1.2 Fill the chromatography column (5.1) to half its height with light petroleum (4.2). Rapidly weigh 22 g of alumina (4.7) into a small beaker and transfer the alumina immediately to the column, promoting settling of the alumina by gently tapping the column.

8.1.3 Add anhydrous sodium sulfate (4.6) to the top of the column to form a layer about 30 mm deep.

8.1.4 Open the tap and allow the light petroleum to fall to the level of the top of the sodium sulfate layer.

8.1.5 Place a 20 ml graduated flask under the column.

8.1.6 Pipette 2,00 ml of the oil solution (8.1.1) onto the column. Rinse the column with minimal amounts of light petroleum, allowing the solvent layer to run into the sodium sulfate layer between rinsings.

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2) Lichrosorb RP-18 is an example of a product commercially available. This information is given for the convenience of users of this International Standard and does not consitute an endorsement by ISO of this product.

3) Chromspher 5 PAH and Vydac 201 TP5 are examples of products commercially available. This information is given for the convenience of users of this International Standard and does not consitute an endorsement by ISO of these products.

8.1.7 Elute the column with light petroleum (4.2) with a flow of about 1 ml/min, discarding the first 20 ml of eluate and collecting the next 60 ml of eluate in a 100 ml round-bottomed flask.

8.1.8 Evaporate the eluate in the water bath (5.2) set at 65 °C, to about 0,5 ml to 1,0 ml, and transfer the concentrated solution into a pre-weighed (to nearest 0,1 mg) crimp-top minivial (5.5).

8.1.9 Continue the evaporation from the minivial, in the water bath (5.2) set at 35 °C under a gentle stream of nitrogen (about 25 ml/min) until nearly dry. Rinse the round-bottomed flask with about 1 ml of light petroleum and transfer the rinsing quantitatively to the minivial, continuing the evaporation under nitrogen. Repeat the rinsing and transfer to the minivial once more.

8.1.10 Continue the evaporation at 35 °C under nitrogen until dry.

8.1.11 Weigh the minivial to the nearest 0,1 mg, and calculate the mass of the residue.

8.1.12 Stopper the minivial with the Teflon-layered septum and aluminium cap and store at 4 °C.

8.2 High-performance liquid chromatography

8.2.1 Use a mixture of 88/12 (*V/V*) acetonitrile (4.3)/water (4.1) as elution solvent. Degas the elution solvent to remove oxygen in order to avoid fluorescence quenching. Use helium purging or an on-line vacuum degasser.

8.2.2 Elute at a flow rate of about 1 ml/min.

8.2.3 Prepare four dilutions of the standard benzo[a]pyrene solutions (4.9.2) such that injection of 10 µl of each will give readings corresponding to 0,04 ng, 0,2 ng, 1,0 ng and 2,0 ng of benzo[a]pyrene. From these, construct a four-point calibration curve using the peak areas from the integrator and chart recorder.

8.2.4 Tetrahydrofuran proved to be the optimal solvent for residue analysis of oils and fats following the clean-up procedure (8.1). Injection of volumes in excess of the specified 10 µl will give rise to problems. Do not store the samples in tetrahydrofuran for a prolonged period as benzo[a]pyrene is not stable in this solvent.

8.3 Sample analysis

8.3.1 Inject 20 µl of tetrahydrofuran (4.4) into the minivial containing the cleansed residue (8.1.12). Dissolve the residue by careful swirling, avoiding contact of the solvent with the septum.

With the calibration curve (8.2.3) benzo[a]pyrene levels of 0,1 µg/kg to 10 µg/kg can be determined. For concentrations above 10 µg/kg, the residue solution (8.3.2) should be diluted further with tetrahydrofuran, or a smaller volume than 10 µl (8.3.2) should be injected.

8.3.2 Inject an accurately known volume of about 10 µl of the dissolved residue into the HPLC column and start the chromatogram running. Care should be taken to ensure that not more than 1,5 mg of residue is introduced into the column. If a larger amount of residue is present, the amount of tetrahydrofuran shall be adjusted or the clean-up step shall be repeated.

9 Expression of results

Calculate the benzo[a]pyrene content of the test sample using the following equation:

$$w = \frac{m_s}{m_r} \times \left(\frac{V_2}{V_3} + \frac{V_0}{m_r \times V_1} \right)$$

where

w is the benzo[a]pyrene content, in micrograms per kilogram, of the test sample;

m_s is the mass, in nanograms, of benzo[a]pyrene read from the calibration curve (8.2.3);

m_r is the mass, in milligrams, of the residue in the minivial obtained in 8.1.11;

r is the assumed density, in milligrams per microlitre, of the residue in the minivial obtained in 8.1.11 (r = 0,8 mg/µl);

V_2 is the volume, in microlitres, of tetrahydrofuran added to the vial in 8.3.1(V_2 = 20 µl);

V_3 is the volume, in microlitres, of tetrahydrofuran injected into the chromatograph;

V_0 is the volume, in millilitres, in which the oil is dissolved (V_0 = 10 ml);

V_1 is the volume, in millilitres, introduced into the aluminium oxide column (V_1 = 2 ml);

m is the mass, in grams, of the test portion.

Express the results to the nearest 0,1 µg/kg .

10 Precision

10.1 Repeatability

The absolute difference between two independent single test results, obtained using 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 be greater than the repeatability limit (*r*) given in table A.1.

10.2 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, will in not more than 5 % of cases be greater than the reproducibility limit (*R*) given in table A.1.

11 Test report

The test report shall specify

all information necessary for the complete identification of the sample;

the sampling method used, if known;

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

all operating details not specified in this International Standard, or regarded as optional, together with any incidents which may have influenced the test result;

the test result obtained;

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

Annex A
(informative)

Results of interlaboratory test

An interlaboratory test, carried out at the international level in 1989 by the International Union of Pure and Applied Chemistry (IUPAC) with 23 laboratories participating, each obtaining two test results on each sample, gave the statistical results (evaluated in accordance with ISO 5725)[4] shown in table A.1. The samples were spiked with known levels of benzo[a]pyrene.

Table A.1 — Statistical results

Sample batch	Fish oil			Rapeseed oil		
	A	B	C	A	B	C
No. of laboratories remaining after eliminating outliers	10	10	10	11	11	11
No. of outliers (labs)	2	2	2	1	1	1
No. of accepted results	240	240	240	264	264	264
Mean value (µg/kg sample)	4,87	3,08	0,98	4,39	2,78	0,99
True or accepted value (µg/kg)	5,35	3,45	1,15	4,85	2,95	1,15
Repeatability standard deviation s_r (µg/kg)	0,64	0,20	0,24	0,73	0,35	0,27
Repeatability coefficient of variability, %	13,1	6,5	24,6	16,6	12,6	27,4
Repeatability limit r (2,8 s_r)	1,79	0,56	0,67	2,04	0,98	0,76
Reproducibility standard deviation s_R (µg/kg)	1,09	0,54	0,29	0,77	0,67	0,41
Reproducibility coefficient of variability, %	22,4	17,6	29,4	17,5	24,0	41,0
Reproducibility limit R (2,8 s_R)	3,05	1,52	0,80	2,16	1,87	1,14

NOTE 1 The repeatability is better for the rapeseed oil than for the fish oil. The repeatability for the fish oil is comparable to the value obtained during the preliminary study within one industrial organization (10 laboratories), reported separately.

NOTE 2 According to reference [3], for an analytical method to be acceptable, the relative reproducibility should be about 45 % at the 1 µg/kg level, and 35 % at the 5 µg/kg level. The range of this criterion is fully met by the results in this study.

4) ISO 5725:1986 (now withdrawn) was used to obtain the precision data.

Annex B
(informative)

Bibliography

[1] ISO 5555:1991, Animal and vegetable fats and oils — Sampling.

[2] ISO 5725:1986, Precision of test methods — Determination of repeatability and reproducibility for a standard test method by inter-laboratory tests.

[3] Horowitz, Report of Collaborative Study. Pure and Applied Chemistry, **63** (11), 1992, pp. 1659-1666.

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