

Methods of analysis of fats and fatty oils —

Part 2: Other methods —

Section 2.47: Determination of benzo[*a*]pyrene content by reverse-phase high-performance liquid chromatography

ICS 67.200.10

National foreword

This British Standard reproduces verbatim ISO 15302:1998 and implements it as the UK national standard.

The UK participation in its preparation was entrusted to Technical Committee AW/11, Animal and vegetable fats and oils, which has the responsibility to:

- aid enquirers to understand the text;
- present to the responsible international/European committee any enquiries on the interpretation, or proposals for change, and keep the UK interests informed;
- monitor related international and European developments and promulgate them in the UK.

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

Cross-references

The British Standards which implement international or European publications referred to in this document may be found in the BSI Standards Catalogue under the section entitled "International Standards Correspondence Index", or by using the "Find" facility of the BSI Standards Electronic Catalogue.

A British Standard does not purport to include all the necessary provisions of a contract. Users of British Standards are responsible for their correct application.

Compliance with a British Standard does not of itself confer immunity from legal obligations.

Summary of pages

This document comprises a front cover, an inside front cover, the ISO title page, page ii, pages 1 to 7 and a back cover.

Amendments issued since publication

Amd. No.	Date	Text affected

This British Standard, having been prepared under the direction of the Consumer Products and Services Sector Board, was published under the authority of the Standards Board and comes into effect on 15 August 1998

© BSI 1998

INTERNATIONAL
STANDARD

ISO
15302

First edition
1998-07-01

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

*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*



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 equilibrate 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.

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 constitute 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 constitute 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:

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

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;
- ρ is the assumed density, in milligrams per microlitre, of the residue in the minivial obtained in 8.1.11 ($\rho = 0,8 \text{ mg}/\mu\text{l}$);
- V_2 is the volume, in microlitres, of tetrahydrofuran added to the vial in 8.3.1 ($V_2 = 20 \mu\text{l}$);
- 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 ($V_0 = 10 \text{ ml}$);
- V_1 is the volume, in millilitres, introduced into the aluminium oxide column ($V_1 = 2 \text{ ml}$);
- m is the mass, in grams, of the test portion.

Express the results to the nearest $0,1 \mu\text{g}/\text{kg}$.

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 ($\mu\text{g/kg}$ sample)	4,87	3,08	0,98	4,39	2,78	0,99
True or accepted value ($\mu\text{g/kg}$)	5,35	3,45	1,15	4,85	2,95	1,15
Repeatability standard deviation s_r ($\mu\text{g/kg}$)	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 ($\mu\text{g/kg}$)	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 $\mu\text{g/kg}$ level, and 35 % at the 5 $\mu\text{g/kg}$ 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.

BSI — British Standards Institution

BSI is the independent national body responsible for preparing British Standards. It presents the UK view on standards in Europe and at the international level. It is incorporated by Royal Charter.

Revisions

British Standards are updated by amendment or revision. Users of British Standards should make sure that they possess the latest amendments or editions.

It is the constant aim of BSI to improve the quality of our products and services. We would be grateful if anyone finding an inaccuracy or ambiguity while using this British Standard would inform the Secretary of the technical committee responsible, the identity of which can be found on the inside front cover. Tel: 0181 996 9000. Fax: 0181 996 7400.

BSI offers members an individual updating service called PLUS which ensures that subscribers automatically receive the latest editions of standards.

Buying standards

Orders for all BSI, international and foreign standards publications should be addressed to Customer Services. Tel: 0181 996 7000. Fax: 0181 996 7001.

In response to orders for international standards, it is BSI policy to supply the BSI implementation of those that have been published as British Standards, unless otherwise requested.

Information on standards

BSI provides a wide range of information on national, European and international standards through its Library and its Technical Help to Exporters Service. Various BSI electronic information services are also available which give details on all its products and services. Contact the Information Centre. Tel: 0181 996 7111. Fax: 0181 996 7048.

Subscribing members of BSI are kept up to date with standards developments and receive substantial discounts on the purchase price of standards. For details of these and other benefits contact Membership Administration. Tel: 0181 996 7002. Fax: 0181 996 7001.

Copyright

Copyright subsists in all BSI publications. BSI also holds the copyright, in the UK, of the publications of the international standardization bodies. Except as permitted under the Copyright, Designs and Patents Act 1988 no extract may be reproduced, stored in a retrieval system or transmitted in any form or by any means – electronic, photocopying, recording or otherwise – without prior written permission from BSI.

This does not preclude the free use, in the course of implementing the standard, of necessary details such as symbols, and size, type or grade designations. If these details are to be used for any other purpose than implementation then the prior written permission of BSI must be obtained.

If permission is granted, the terms may include royalty payments or a licensing agreement. Details and advice can be obtained from the Copyright Manager. Tel: 0181 996 7070.