INTERNATIONAL STANDARD

ISO 15302

Second edition
2007-12-15

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

Corps gras d'origines animale et végétale — Détermination du benzo[a]pyrène — Méthode par chromatographie liquide à haute performance à polarité de phase inversée

Reference number
ISO 15302:2007(E)

PDF disclaimer

This PDF file may contain embedded typefaces. In accordance with Adobe's licensing policy, this file may be printed or viewed but shall not be edited unless the typefaces which are embedded are licensed to and installed on the computer performing the editing. In downloading this file, parties accept therein the responsibility of not infringing Adobe's licensing policy. The ISO Central Secretariat accepts no liability in this area.

Adobe is a trademark of Adobe Systems Incorporated.

Details of the software products used to create this PDF file can be found in the General Info relative to the file; the PDF-creation parameters were optimized for printing. Every care has been taken to ensure that the file is suitable for use by ISO member bodies. In the unlikely event that a problem relating to it is found, please inform the Central Secretariat at the address given below.

COPYRIGHT PROTECTED DOCUMENT

ISO copyright office
Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

Published in Switzerland

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.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

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

This second edition cancels and replaces the first edition (ISO 15302:1998).

Animal and vegetable fats and oils — Determination of benzo[*a*]pyrene — 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 0,1 µg/kg to 50 µg/kg.

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
benzo[*a*]pyrene content
mass fraction of benzo[*a*]pyrene in the test portion, as determined using the method specified in this International Standard

NOTE The content is expressed in micrograms per kilogram.

4 Principle

A test portion is dissolved in light petroleum and benzo[*b*]chrysene is added as internal standard. A suitable amount of sample is adsorbed on an alumina column, and eluted with light petroleum to remove any benzo[*a*]pyrene present.

5 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified. 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.

SAFETY PRECAUTIONS — Attention is drawn to regulations which specify handling procedures for dangerous substances. Users should be aware of and comply with technical, organizational, and personal safety measures.

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

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

5.3 Acetonitrile, suitable for HPLC.

5.4 Tetrahydrofuran, suitable for HPLC.

5.5 Acetonitrile-tetrahydrofuran mixture, prepared by mixing 90 ml acetonitrile (5.3) and 10 ml tetrahydrofuran (5.4).

5.6 Toluene, suitable for HPLC.

5.7 Sodium sulfate, granular, anhydrous.

5.8 Alumina activity grade 4, prepared from neutral aluminium oxide, activity grade super 1[1)], deactivated by the addition of 10 ml water (5.1) to 90 g of alumina.

Due to the differences in activity of alumina of various brands, a check is recommended to confirm that the deactivation procedure is appropriate for total benzo[*a*]pyrene recovery from a reference sample.

CAUTION — THE DEACTIVATION 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.

5.9 Benzo[*a*]pyrene[2)], of purity 99,0 % by mass.

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

5.9.1 Benzo[*a*]pyrene stock solution in toluene, 0,5 mg/ml.

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 (5.6) and make up to the mark with that solvent.

Store the solution in the dark at 4 °C where it is stable for at least 6 months.

5.9.2 Benzo[*a*]pyrene 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 (5.9.1) with acetonitrile.

1) "Aluminium oxide 90 active neutral" is an example of a suitable product available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

2) A suitable reference material is available from the Joint Research Centre of the European Commission, Institute for Reference Materials and Measurements (IRMM). This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

5.10 Benzo[*b*]chrysene[3] internal standard solution in acetonitrile.

Prepare a stock solution containing, to the nearest nanogram, approximately 10 ng/µl. Dilute this solution by a factor of 10 in a volumetric flask, to obtain an internal standard solution with a concentration of approximately 1 ng/µl.

NOTE This solution may also be prepared by dissolving benzo[*b*]chrysene[3], 99,0 % by mass, in acetonitrile.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

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

6.2 Water baths, one maintained at (35 ± 2) °C and another at (65 ± 2) °C.

6.3 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.

6.4 High performance liquid chromatograph, consisting of an HPLC pump, injection valve with 50 µl sample loop, reverse-phase column, electronic integrator and chart recorder.

If an autosampler is used, the sample loop shall be flushed with acetonitrile between consecutive injections.

6.5 Columns for HPLC analysis

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

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

6.6 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.

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

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

6.9 Disposable pipettes.

3) A suitable reference material is available from the Joint Research Centre of the European Commission, Institute for Reference Materials and Measurements (IRMM) or Dr. Ehrenstorfer GmbH. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product.

4) Examples of suitable products available commercially. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of this product. Equivalent products may be used if they can be shown to lead to the same results.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have been damaged or changed during transport or storage.

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

8 Preparation of test sample

Prepare the test sample in accordance with ISO 661.

9 Procedure

9.1 Clean up of sample

9.1.1 Weigh, to the nearest 0,001 g, about 0,400 g of the fat or oil into a glass beaker and dissolve in 2 ml of light petroleum (5.2). Add 20 µl of the internal standard solution (5.10) by means of a microsyringe. This is equivalent to 50 µg/kg when calculated on the sample mass. If a high level of benzo[*a*]pyrene is expected, then add 50 µl of the internal standard solution (5.10). This is equivalent to 125 µg/kg when calculated on the sample mass.

9.1.2 Fill the chromatography column (6.1) to half its height with light petroleum (5.2). Rapidly weigh 22 g of alumina (5.8) into a small beaker and transfer the alumina immediately to the column, then gently tap the column to effect settling of the alumina.

9.1.3 Add anhydrous sodium sulfate (5.7) to the top of the column to form a layer about 30 mm deep.

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

9.1.5 Place a 20 ml graduated flask under the column.

9.1.6 Introduce the oil solution (9.1.1) on to the column. Rinse the column with minimal amounts of light petroleum (5.2), allowing the solvent layer to run into the sodium sulfate layer between rinsings.

9.1.7 Elute the column with light petroleum 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.

9.1.8 Evaporate solvent from the eluate in the water bath set at 65 °C, to a volume of about 0,5 ml to 1,0 ml, and transfer the concentrated solution into a crimp-top minivial (6.7) pre-weighed to the nearest 0,1 mg.

9.1.9 Continue the evaporation from the minivial, in the water bath (5.1) 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.

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

9.1.11 Weigh the minivial to the nearest 0,1 mg, and calculate the mass of the residue. Stopper the minivial with the PTFE-layered septum and aluminium cap and store at 4 °C.

9.2 High performance liquid chromatography

9.2.1 Use a mixture of 880 ml acetonitrile (5.3) and 120 ml water (5.1) 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.

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

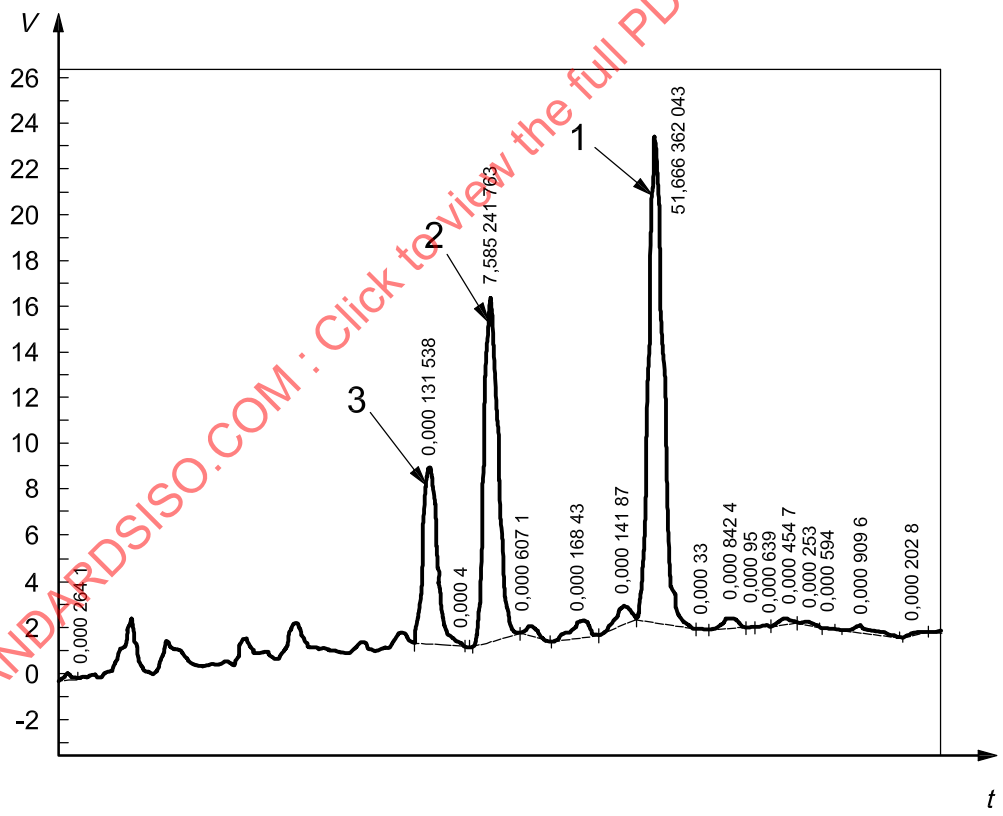
9.2.3 Calibration curve and determination of the relative response factor: Prepare five dilutions of the standard benzo[*a*]pyrene solutions (5.9.2) such that injection of 50 µl of each will give readings corresponding to 0,01 ng, 0,04 ng, 0,2 ng, 1,0 ng and 2,0 ng of benzo[*a*]pyrene. Add to the standard solutions 0,5 ng of internal standard. From these, construct a five-point calibration curve using the peak areas from the integrator and chart recorder. These calibrations are also used to calculate the relative response factor (10.1), between benzo[*a*]pyrene and the internal standard.

9.3 Sample analysis

9.3.1 Inject 250 µl of the acetonitrile-tetrahydrofuran mixture (5.5) into the minivial containing the cleansed residue (9.1.11). Dissolve the residue by careful swirling, avoiding contact of the solvent with the septum.

With the calibration curve (9.2.3), benzo[*a*]pyrene levels of 0,1 µg/kg to 50 µg/kg can be determined. For concentrations above 10 µg/kg, the residue solution (9.3.2) should be diluted further with acetonitrile-tetrahydrofuran (5.5), or a smaller volume than 50 µl (9.3.2) should be injected.

9.3.2 Inject an accurately known volume of about 50 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 (5.4) shall be adjusted or the clean-up step shall be repeated.



Key

V HPLC absorbance/mV
t time/min
1 benzo[*b*]chrysene (internal standard)
2 benzo[*a*]pyrene
3 benzo[*k*]fluoranthene

Figure 1 — Typical chromatogram

10 Expression of results

10.1 Calculation of relative response factors

From the data obtained (9.2.3), calculate the relative response factor, f_{rr}, as the arithmetic average of five standards, using Equation (1):

$$f_{rr} = \frac{A_{is}\rho_{BaP}}{A_{BaP}\rho_{is}} \quad (1)$$

where:

A_{BaP} is the peak area of benzo[*a*]pyrene;

A_{is} is the peak area of the internal standard solution;

ρ_{BaP} is the concentration, in micrograms per litre, of benzo[*a*]pyrene;

ρ_{is} is the concentration, in micrograms per litre, of the internal standard solution.

10.2 Calculation of benzo[*a*]pyrene content

Calculate the benzo[*a*]pyrene content, w_{BaP}, in micrograms per kilogram, of the test sample using Equation (2):

$$w_{BaP} = \frac{A_{BaP}}{A_{is}}\rho_{is}\frac{1}{m}f_{rr} \quad (2)$$

where

f_{rr} is the average response factor, calculated from the standard solutions (10.1);

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

ρ_{is} is the concentration, in nanograms per litre, of the internal standard added to the eluate.

The content is expressed as a mass fraction, in micrograms per kilogram, to one decimal place for contents of 0 µg/kg to 10 µg/kg and to the nearest integer for contents greater than 10 µg/kg.

11 Precision

11.1 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.

11.2 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.

12 Test report

The test report shall specify at least the following information:

a) all the information required 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 incident that may have influenced the result(s);

e) the test result(s) obtained;

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