
**Animal and vegetable fats and oils —
Determination of polycyclic aromatic
hydrocarbons**

*Corps gras d'origines animale et végétale — Détermination des
hydrocarbures aromatiques polycycliques*

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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 15753 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

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Animal and vegetable fats and oils — Determination of polycyclic aromatic hydrocarbons

1 Scope

This International Standard describes two methods for the determination of 15 polycyclic aromatic hydrocarbons (PAHs) in animal and vegetable fats and oils:

- a general method, and
- a method specific for coconut oil and vegetable oils with short-chain fatty acids.

These methods are not quantitative for the very volatile compounds such as naphthalene, acenaphthene and fluorene. Due to interferences provided by the matrix itself, palm oil and olive pomace oil cannot be analysed using this method.

The quantification limit is 0,2 µg/kg for almost all compounds analysed, except for fluoranthene and benzo(*g,h,i*)perylene where the quantification limit is 0,3 µg/kg, and indeno(1,2,3-*c,d*)pyrene where the quantification limit is 1 µg/kg.

2 Normative references

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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

polycyclic aromatic hydrocarbon

PAH

compound that contains two or more condensed (fused) aromatic hydrocarbon rings and the content of which can be determined according to the method specified in this International Standard

NOTE 1 The content is given in micrograms per kilogram.

NOTE 2 In general PAHs are divided into light PAHs with two to four aromatic rings, and heavy PAHs with five or more aromatic rings.

EXAMPLES

Light PAHs include:

naphthalene (CAS RN [91-20-3]), acenaphthene (CAS RN [83-32-9]), acenaphthylene (CAS RN [208-96-8]), fluorene (CAS RN [86-73-7]), anthracene (CAS RN [120-12-7]), phenanthrene (CAS RN [85-01-8]), fluoranthene (CAS RN [206-44-0]), chrysene (CAS RN [218-01-9]), benz(*a*)anthracene (CAS RN [56-55-3]), pyrene (CAS RN [129-00-0]).

Heavy PAHs include:

benzo(a)pyrene (CAS RN [50-32-8]), benzo(b)fluoranthene (CAS RN [205-99-2]), benzo(k)fluoranthene (CAS RN [207-08-9]), benzo(g,h,i)perylene (CAS RN [191-24-2]), dibenz(a,h)anthracene (CAS RN [53-70-3]), indeno(1,2,3-c,d)pyrene (CAS RN [193-39-5]).

4 Principle

The polycyclic aromatic hydrocarbons are extracted with an acetonitrile/acetone mixture followed by purification on C18 reversed-phase and then Florisil bonded-phase cartridges. Determination of the content of the individual polycyclic aromatic hydrocarbons after separation is achieved by means of high-pressure liquid chromatography (HPLC) and by measuring the fluorescence at various excitation and emission wavelengths.

5 Reagents and materials

WARNING — Attention is drawn to the regulations governing the handling of dangerous matter. Technical, organizational and personal safety measures must be followed.

Use only reagents of recognized analytical grade unless otherwise stated.

Check the quality of solvents before use by concentrating the solvent about 1 000 times by evaporation and analysing the concentrate by HPLC (300 ml to 300 µl). The chromatogram shall be free from peaks in the elution area of PAHs.

5.1 **Methanol**, 'ultra resi-analysed' grade¹⁾.

5.2 **Hexane**, HPLC grade¹⁾.

5.3 **Acetonitrile**, HPLC grade¹⁾.

5.4 **Acetone**, HPLC grade¹⁾.

5.5 **Dichloromethane**, HPLC grade¹⁾.

5.6 **Toluene**, HPLC grade¹⁾.

5.7 **Water**, HPLC grade¹⁾.

5.8 **Tetrahydrofuran**, HPLC grade¹⁾.

5.9 **Solvent mixture 1**: acetonitrile/acetone (volume fraction 60 % / 40 %).

Quantity used per sample: 41 ml for general method, 36 ml for method specific for coconut oil.

5.10 **Solvent mixture 2**: acetonitrile/acetone (volume fraction 80 % / 20 %).

Quantity used per sample: 2 × 11 ml for method specific for coconut oil.

5.11 **Solvent mixture 3**: hexane/dichloromethane (volume fraction 75 % / 25 %).

Quantity used per sample: 7 ml for general method, 2 × 7 ml for method specific for coconut oil.

1) These can be obtained from, for example, Baker.

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5.12 Mixture of tetrahydrofuran/methanol (volume fraction 50 % / 50 %).

5.13 Standard solution with 16 certified EPA Priority PAHs in toluene²⁾, at a concentration of 100 µg/ml (100 mg/l): naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenz(a,h)anthracene, benzo(g,h,i)perylene, indeno(1,2,3-c,d)pyrene. To be stored at -20 °C.

Before use, allow the solution to warm up to ambient temperature for at least 1 h.

NOTE Acenaphthylene is not fluorescent and, thus, it cannot be determined by these methods.

5.14 Stock standard solution, 200 ng/ml (200 µg/l).

Add 100 µl of standard solution (5.13) with a 250 µl syringe (6.11) to a 50 ml volumetric flask (6.20) and dilute to the mark with acetonitrile.

5.15 Working standard solution, 50 ng/ml (50 µg/l).

Add 250 µl of stock standard solution (5.14) with a 250 µl syringe (6.11) to 750 µl of THF/methanol mixture (5.12) or acetonitrile (5.3).

5.16 C18 bonded-phase cartridges³⁾, 2 g phase, 12 ml capacity.

5.17 Florisil bonded-phase cartridges³⁾, 500 mg phase, 3 ml capacity.

5.18 Stream of nitrogen, pressure regulated at 34,5 kPa (5 psi) about 1,5 l/min).

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6 Apparatus

Usual laboratory apparatus and, in particular, the following
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The use of disposable glass tubes is acceptable. The general use of glass is necessary as plastics can contain PAHs.

6.1 Centrifuge, capable of attaining at least 4 000 min⁻¹, suitable for 100 ml and 10 ml tubes.

6.2 HPLC system with binary gradient elution, with solvent reservoir of 1 l capacity, a mobile phase liner filter, pump, autosampler, column temperature regulation set at 25 °C, fluorescence detector programmable over time for various excitation and emission wavelengths, and computer-assisted acquisition and data treatment.

6.3 C18 reversed-phase column⁴⁾, 250 mm in length, 4,6 mm internal diameter, 5 µm particles, suitable for PAH analysis.

6.4 Vortex mixer.

6.5 Automatic evaporator⁵⁾, for 10 ml tube (optional), or water bath (6.6).

2) This can be obtained from, for example, Promochem.

3) This can be obtained from, for example, Varian.

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4) This can be obtained from, for example, Vydac, ref. 201TP54.

5) This can be obtained from, for example, Zymark, Zymark TurboVap LV evaporator.

Recommended operating conditions:

- temperature of water bath 35 °C;
- nitrogen pressure 34,5 kPa.

6.6 Water bath, regulated at 35 °C.

6.7 Balance, with readability of 0,1 mg.

6.8 Centrifuge tubes, of 100 ml capacity (one per sample).

6.9 Conical centrifuge tubes, of 11 ml capacity (three per sample), with PTFE septa and closed top screw caps (one per sample).

6.10 Graduated cylinders.

6.11 Microsyringe, 250 µl.

6.12 Syringe, 1 000 µl.

6.13 Graduated pipette, 5 ml.

6.14 Syringe, 5 ml, equipped with an adapter cap for SPE cartridges.

6.15 Vials for autosampler.

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6.16 Microvials, of 250 µl capacity, adapted for HPLC system.

6.17 Ultrasonic bath, with water temperature not higher than 40 °C.

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6.18 Pasteur pipettes, with cotton wool in the top part to prevent contamination.

6.19 Device composed of stand and pincers⁶⁾, to hold SPE cartridges or, if available, an automatic SPE work station.

NOTE Depending on the SPE sample processing station used, the proposed extraction methods may require slight adaptations (times, pressure, volumes).

6.20 Volumetric flask, of capacity 50 ml.

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.

6) This can be obtained from, for example, Zymark, Zymark Rapid Trace.

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8 Sample preparation

Prepare the test sample in accordance with the method given in ISO 661. Before sampling, the liquid samples shall be at room temperature and homogenized by magnetic agitation.

Sample the solid matrix by melting the entire sample or by melting and homogenizing several core samples.

9 Procedure for determination of PAHs from fats and oils: General method

9.1 Preliminary remarks

In order to obtain repeatable results, the ambient temperature of the laboratory shall be regulated (≤ 20 °C). This is a very important condition for the extraction of PAHs from coconut oil (or vegetable oils containing short-chain fatty acids). These oils contain fatty acids with short and long chains; when the ambient temperature is higher than 20 °C, the solubility of short-chain fatty acids increases.

Before use, rinse the whole vessel three times with hexane (5.2).

Each sequence of samples shall include a blank (9.2), and a standard solution extracted under the same conditions as the sample in order to calculate the recovery values of the extraction (9.3). The recovery values shall be within the range 70 % to 110 %. The mean recovery values are given in Table A.1.

For a quantitative analysis, two test portions shall be extracted and analysed separately, the final result being the mean value of the results of these two subsamples.

It is not possible to complete the entire analysis within a single day. Sample extracts shall be stored overnight under deep-freeze conditions of at least -18 °C:

- 1st day: step 1, step 2 and step 3, up to purification on C18 cartridge (see Figure A.1).
- 2nd day: step 3, purification on Florisil cartridge and preparation of HPLC system for sample analysis (see Figure A.1).
- following night and day(s): analysis of the samples (see Table A.2).

9.2 Blank

To ensure the absence of contamination of solvents and cartridges, the purification procedure (according to 9.5, 9.6 and Clause 11) shall first be carried out on a blank sample (sample with solvent mixture but with the oil omitted). The chromatogram obtained shall be free from the compounds of interest. If the chromatogram contains interferences, the source of interferences shall be determined and eliminated. Blank values cannot be used to correct sample values as blank values are generally not homogenous (repeatability).

9.3 Determination of recovery values (without matrix)

In order to verify the extraction efficiency of cartridges, carry out a test with a standard solution. Spike 1 750 μ l of solvent mixture 1 (5.9) with 250 μ l of working standard solution (5.15) with a 250 μ l syringe (6.11). Transfer to a C18 cartridge and treat as described in 9.5, 9.6 and Clause 11).

WARNING — When removing solvents under a stream of nitrogen (see 9.5.6), do not evaporate to dryness but leave about 50 μ l in the vial, otherwise volatile PAHs will be lost.

9.4 Extraction (liquid/liquid extraction)

9.4.1 The flow chart of the isolation procedure is given in Figure A.1.

9.4.2 Weigh, to the nearest 1 mg, about 2,5 g of the sample into a 100 ml centrifuge tube (6.8). Add 10 ml of solvent mixture 1 (5.9).

9.4.3 Agitate the centrifuge tube for 30 s with the vortex mixer (half speed), and then put the tube in an ultrasonic bath (6.17) for 5 min.

9.4.4 Centrifuge for 5 min at 4 000 min⁻¹.

9.4.5 Carefully remove the top layer with a Pasteur pipette (6.18) and transfer it to a weighed conical tube (6.9).

9.4.6 Evaporate the solvent from the conical tube for 30 min to 40 min, under a stream of nitrogen (5.18), using either a water bath at 35 °C (6.6) or an automatic evaporator (6.5).

9.4.7 Repeat the extraction twice with a further 10 ml of solvent mixture 1 (5.9). Concentrate the extracts in the same conical tube under a stream of nitrogen (5.18) using water bath set at 35 °C (6.6) or using an automatic evaporator (6.5). The fat residue should be about 200 mg to 800 mg.

If the fat residue mass is higher than 800 mg, then the general method (Clause 9) is not suitable and the method specific for coconut oil should be used (Clause 10).

9.5 Purification on C18-bonded phase cartridge (solid/liquid extraction)

9.5.1 Cartridge conditioning: Put the cartridge (5.16) on a stand (6.19). Rinse the cartridge with 2 volumes of 12 ml of methanol (5.1) then 2 volumes of 12 ml of acetonitrile (5.3). Allow the solvent to flow through under atmospheric pressure.

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9.5.2 Put a weighed conical tube (6.9) under the cartridge (5.16).

9.5.3 With a syringe (6.12) or a graduated pipette (6.13), introduce 2 ml of solvent mixture 1 (5.9) into the conical tube containing residual fat material (9.4.6). Agitate the tube with the vortex mixer (6.4) for 15 s. Centrifuge for 30 s. Transfer the top layer to the cartridge (5.16) with a Pasteur pipette (6.18). Repeat the operation twice (2 ml of solvent mixture 1, mixing, centrifuging and transferring onto the cartridge). Collect the solvent eluting from the cartridge together with the elution solvent.

9.5.4 Add 5 ml of solvent mixture 1 (5.9) to the top of the cartridge (5.16) and allow the elution to proceed under atmospheric pressure.

9.5.5 Using a syringe (6.14), inject air into the cartridge in order to elute the remaining solvent and any PAHs which could be retained in the phase.

9.5.6 Remove solvents under a stream of nitrogen (5.18) using a water bath set at 35 °C (6.6) or an automatic evaporator (6.5). The fat residue should be not more than 50 mg.

9.5.7 Dilute the residue in 1 ml of hexane (5.2), measured with a syringe (6.12). Close the conical tube hermetically and store at -18 °C until further use.

9.6 Purification on Florisil-bonded phase cartridge (solid/liquid extraction)

9.6.1 Allow the extract (9.5.7) to warm up to ambient temperature for at least 1 h.

9.6.2 Cartridge conditioning: Put the cartridge (5.17) on a stand (6.19). Rinse the cartridge with 5 volumes of 3 ml of dichloromethane (5.5) then 4 volumes of 3 ml of hexane (5.2).

9.6.3 Put a weighed conical tube (6.9) under the cartridge (5.17).

9.6.4 Transfer the extract (9.5.7) to the cartridge (5.17) with a Pasteur pipette (6.18).

9.6.5 Introduce 1 ml of solvent mixture 3 (5.11), using a syringe (6.12) or a graduated pipette (6.13), to the conical tube containing the extract. Agitate it for 15 s with the vortex mixer and transfer it to the cartridge (5.17). Rinse the tube with 2×2 ml of solvent mixture 3 (5.11) and transfer it onto the cartridge. Collect the solvent eluting from the cartridge together with the elution solvent.

Pay careful attention to avoid contact between the pipette and the conical tube in order to prevent cross contamination.

9.6.6 Elute 4 ml of solvent mixture 3 (5.11) through the cartridge (5.17). Using a syringe (6.14), inject air into the cartridge in order to elute the remaining solvent.

9.6.7 Concentrate the solution under a stream of nitrogen (5.18), using a water bath set at 35 °C (6.6) or an automatic evaporator (6.5), to about 1 ml (takes 10 min to 15 min). Add about 0,5 ml of toluene (5.6) (keeper) and continue to evaporate until about 50 µl remain.

The solvent should not be removed completely.

9.6.8 The exact volume is determined by weighing the conical tube, and calculating using the density of toluene. Add the necessary volume of solvent [MeOH/THF (5.12), or acetonitrile (5.3)], V_{add} , to make up to 250 µl:

$$V_{\text{add}} = 250 - \frac{m}{d}$$

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where

m is the sample mass, in milligrams; [ISO 15753:2006](https://standards.iteh.ai/catalog/standards/sist/2a446d71-c393-4df6-910a-0007727c5e/iso-15753-2006)

d is the density of toluene (0,866 9 kg/m³); <https://standards.iteh.ai/catalog/standards/sist/2a446d71-c393-4df6-910a-0007727c5e/iso-15753-2006>

9.6.9 Transfer the sample to a microvial (6.16) placed in a vial (6.15).

10 Procedure for determination of PAHs from fats and oils: Method specific for coconut oil

10.1 First extraction (liquid/liquid extraction)

10.1.1 The flow chart of the isolation procedure is given in Figure A.2.

10.1.2 Weigh, to the nearest 1 mg, about 2 g of the sample into a 100 ml centrifuge tube (6.8). Add 10 ml of solvent mixture 1 (5.9).

10.1.3 Agitate the centrifuge tube for 30 s with the vortex mixer (half speed), and then put the tube in an ultrasonic bath (6.17) for 5 min.

10.1.4 Centrifuge for 5 min at 4 000 min⁻¹.

10.1.5 Carefully remove the top layer with a Pasteur pipette (6.18) and introduce it into a conical tube (6.9).

10.1.6 Evaporate the solvent from the conical tube for 30 min to 40 min under a stream of nitrogen (5.18) using the water bath set at 35 °C (6.6) or an automatic evaporator (6.5).