
**Animal and vegetable fats and oils —
Determination of polycyclic aromatic
hydrocarbons by on-line donor-acceptor
complex chromatography and HPLC with
fluorescence detection**

*Corps gras d'origines animale et végétale — Détermination de la teneur
en hydrocarbures aromatiques polycycliques par chromatographie de
complexe donneur-accepteur et CLHP avec détection par fluorescence*

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Published in Switzerland

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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 22959 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

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Introduction

Polycyclic aromatic hydrocarbons (PAHs) are formed during pyrolytic processes such as the incomplete combustion of organic substances or have a petrogenic origin (mineral oils). Edible fats and oils may be contaminated by environmental pollution and/or processing steps prior to refining. The presence of PAHs in fats and oils is a health concern due to their carcinogenicity. Different levels of PAHs have been observed in crude edible oils. Refining of the oils (deodorization, bleaching, charcoal treatment) under the appropriate conditions reduces the content of the individual PAHs to the microgram per kilogram level. The known methods of analysis of PAHs in edible fats and oils include complex and laborious extraction and clean-up procedures to isolate the low levels of PAHs present.

With the donor-acceptor complex-chromatography (DACC) technique, PAHs can be extracted from different matrices. PAHs are electron donors (π -electrons) and the strong interaction of the PAHs with an electron acceptor stationary phase results in retention of the PAHs and elution of (the bulk of) the other components of the oil. This International Standard specifies an automated on-line method for the determination of PAHs in edible oils and fats, which can easily be applied as a routine analysis. The method consists of an LC-LC coupling of a clean-up DACC column to an analytical column for the separation. PAHs are quantified by fluorescence detection.

Compared to older techniques, this automated on-line method significantly reduces the amount of solvent used and saves considerable time. The DACC column clean-up is fast and is carried out during the HPLC run of the previous sample. The total analysis time for one sample is approximately 90 min, compared with the traditional methods which require 8 h to 10 h. Moreover, the system can run 24 h/day. Finally, losses of volatile PAHs during solvent evaporation, for example, are eliminated. The quantification limits of 0,1 $\mu\text{g}/\text{kg}$ of the individual PAHs have been retained with the DACC method, which automatically corrects for possibly incomplete recoveries because the calibration samples are subjected to the same treatment as the samples to be analysed. The system uses conventional HPLC instrumentation.

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Animal and vegetable fats and oils — Determination of polycyclic aromatic hydrocarbons by on-line donor-acceptor complex chromatography and HPLC with fluorescence detection

1 Scope

This International Standard specifies a high performance liquid chromatographic (HPLC) procedure for the determination of polycyclic aromatic hydrocarbons (PAHs) in edible fats and oils.

The method has been validated for coconut (CN), olive (OV), sunflower (SF), and soybean (BO) oil, and is possibly applicable to other oils, dependent on the determination of appropriate parameters.

The lowest level of quantification for the PAHs is 0,1 µg/kg. The lowest possible amount of each PAH which can be distinguished from the baseline noise has not been determined. The validated concentration range of the method is 0,1 µg/kg to 3,5 µg/kg for each individual PAH. For samples containing (light) PAH contents > 3,5 µg/kg, dilution to bring the contents into the validated range is possible. It is also possible to adjust the range of the calibration curves. However, ranges exceeding 3,5 µg/kg have not been validated.

PAHs which can be determined by this method are: anthracene, phenanthrene, fluoranthene, pyrene, chrysene, benzo[*a*]anthracene, benzo[*e*]pyrene, benzo[*a*]pyrene, perylene, benzo[*ghi*]perylene, anthanthrene, dibenzo[*a,h*]anthracene, coronene, indeno[1,2,3-*cd*]pyrene, benzo[*a*]fluoranthene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene.

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

polycyclic aromatic hydrocarbon

PAH

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

3.2 light polycyclic aromatic hydrocarbon

compound with two to four condensed (fused) aromatic hydrocarbon rings

EXAMPLES

Compound	CAS No.	Compound	CAS No.	Compound	CAS No.
acenaphthene	83-32-9	benzo[e]pyrene	192-97-2	naphthalene	50-32-8
acenaphthylene	208-96-8	chrysene	218-01-9	phenanthrene	85-01-8
anthracene	120-12-7	fluoranthene	206-44-0	pyrene	129-00-0
benzo[a]anthracene (1,2-benzoanthracene)	56-55-3	fluorene	86-73-7		

3.3 heavy polycyclic aromatic hydrocarbon

compound with five and more condensed (fused) aromatic hydrocarbon rings

EXAMPLES

Compound	CAS No.	Compound	CAS No.	Compound	CAS No.
benzo[a]pyrene (1,2-benzopyrene)	50-32-8	benzo[k]fluoranthene	207-08-9	dibenzo[a,h]anthracene (1,2,5,6-dibenzoanthracene)	53-70-3
benzo[a]fluoranthene	203-33-8	benzo[ghi]perylene (1,12-benzoperylene)	191-24-2	indeno[1,2,3-cd]pyrene	193-39-5
benzo[b]fluoranthene	205-99-2	coronene	191-07-1	perylene	198-55-0

3.4 polycyclic aromatic hydrocarbon content

mass fraction of a polycyclic aromatic hydrocarbon or polycyclic aromatic hydrocarbon mixture in a matrix

EXAMPLES Individual polycyclic aromatic hydrocarbon content; light polycyclic aromatic hydrocarbon content; heavy polycyclic aromatic hydrocarbon content.

NOTE The content is expressed as a mass fraction in micrograms per kilogram.

4 Principle

The PAHs in edible oils are determined by on-line coupling of donor-acceptor complex chromatography (DACC) and HPLC with fluorescence detection. The oil samples are eluted over a column with a modified stationary phase (DACC column) which will act as an electron acceptor. This column will retain the PAHs (electron donors) by π - π interactions. After elution of the oil, the PAHs are transferred on-line to the analytical reversed phase column. The individual PAHs are detected at different wavelengths. The retention times of the PAHs are used to identify the individual compounds. The levels of the PAHs in the oil samples are calculated by external calibration.

5 Reagents, materials and standards

WARNING — The method requires harmful reagents. Respect normal laboratory safety regulations. All PAHs are suspected carcinogenic compounds. Therefore, it is essential that the preparation of the stock solutions, the standard dilutions and the samples of the calibration curve (5.3) are performed by preference in a class-2 laboratory. Furthermore, a laboratory coat and safety gloves are essential for these steps. Contaminated tissues and gloves shall be collected in a plastic bag and removed after sealing the bag.

5.1 Reagents.

5.1.1 **Acetonitrile**, HPLC grade, mass fraction $w[\text{C}_2\text{H}_3\text{N}] > 99,9 \%$.

5.1.2 **Ethyl acetate**, HPLC grade, mass fraction $w[\text{C}_4\text{H}_8\text{O}_2] > 99,8 \%$.

5.1.3 **2-Propanol**, HPLC grade, mass fraction $w[\text{C}_3\text{H}_8\text{O}] > 99,9 \%$.

5.1.4 **Toluene**, HPLC grade, mass fraction $w[\text{C}_7\text{H}_8] > 99,9 \%$.

5.1.5 **Water**, HPLC grade.

5.2 Standards.¹⁾

5.2.1 **Anthracene**, mass fraction $w[\text{C}_{14}\text{H}_{10}] > 99 \%$.

5.2.2 **Phenanthrene**, mass fraction $w[\text{C}_{14}\text{H}_{10}] > 99 \%$.

5.2.3 **Fluoranthene**, mass fraction $w[\text{C}_{16}\text{H}_{10}] > 99 \%$.

5.2.4 **Pyrene**, mass fraction $w[\text{C}_{16}\text{H}_{10}] > 99 \%$.

5.2.5 **Chrysene**, mass fraction $w[\text{C}_{18}\text{H}_{12}] > 99 \%$.

5.2.6 **Benzo[a]anthracene** (1,2-Benzoanthracene), mass fraction $w[\text{C}_{18}\text{H}_{12}] > 99 \%$.

5.2.7 **Benzo[e]pyrene**, mass fraction $w[\text{C}_{20}\text{H}_{12}] > 99 \%$.

5.2.8 **Benzo[a]pyrene** (1,2-Benzopyrene), mass fraction $w[\text{C}_{20}\text{H}_{12}] > 99 \%$.

5.2.9 **Perylene**, mass fraction $w[\text{C}_{20}\text{H}_{12}] > 99 \%$.

5.2.10 **Benzo[ghi]perylene** (1,12-Benzoperylene), mass fraction $w[\text{C}_{22}\text{H}_{12}] > 99 \%$.

5.2.11 **Anthanthrene**, mass fraction $w[\text{C}_{22}\text{H}_{12}] > 99 \%$.

5.2.12 **Dibenzo[a,h]anthracene** (1,2,5,6-Dibenzoanthracene), mass fraction $w[\text{C}_{22}\text{H}_{14}] > 99 \%$.

5.2.13 **Coronene**, mass fraction $w[\text{C}_{24}\text{H}_{12}] > 99 \%$.

5.2.14 **Indeno[1,2,3-cd]pyrene**, mass fraction $w[\text{C}_{22}\text{H}_{12}] > 99 \%$.

5.2.15 **Benzo[a]fluoranthene**, mass fraction $w[\text{C}_{20}\text{H}_{12}] > 99 \%$.

5.2.16 **Benzo[b]fluoranthene**, mass fraction $w[\text{C}_{20}\text{H}_{12}] > 99 \%$.

5.2.17 **Benzo[k]fluoranthene**, mass fraction $w[\text{C}_{20}\text{H}_{12}] > 99 \%$.

5.2.18 **BCR certified reference material 458**, coconut oil with 6 PAHs.

1) IRMM (<http://www.irmm.jrc.be>) and Sigma-Aldrich (<http://www.sigmaaldrich.com>) are suitable suppliers. This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of products so supplied. Products from other sources may be used if they can be shown to lead to the same results.

5.3 Standard solutions.

5.3.1 PAH standard solutions in toluene, mass concentration approx. 0,2 mg/ml. Weigh, to the nearest 0,01 mg, approx. 10 mg of all PAHs (5.2.1 to 5.2.17) into separate 50 ml one-mark volumetric flasks (6.7) and make up to the mark with toluene (5.1.4).

5.3.2 PAH standard solution in oil, mass fraction approx. 125 µg/kg. Prepare a PAH standard solution in oil of the same type of oil (5.3.3) as the samples to be analysed.

Transfer, with a syringe (6.2), 10,0 µl of each standard solution (5.3.1) to one 20 ml vial (6.1) with crimp cap. Wait until (most of) the toluene is evaporated and weigh 16 g of oil to the nearest 0,1 mg in the vial. Mix thoroughly.

5.3.3 Preparation of the oils used for standard solutions (blank and dilutions). Weigh approximately 400 g of (preferably) refined oil into a 1 l round-bottomed flask. Add 20 g of activated charcoal²⁾. Heat for 2 h at 90 °C in a rotary evaporator under vacuum, centrifuge the mixture and filter the supernatant over a 0,45 µm filter (6.3).

Analyse the oil to check whether the background of PAHs is much smaller than 0,1 µg/kg. If necessary, the level of the light PAHs can be lowered by steaming for approx. 3 h at 240 °C with 3 % volume fraction steam/hour at a pressure lower than 3 kPa.

5.3.4 Samples for PAH calibration curve. The calibration curve samples are prepared for the same type of oil as the samples to be analysed. The background of PAHs in the oil used should be much smaller than 0,1 µg/kg.

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Prepare six calibration samples by weighing different amounts of the PAH standard solution in oil (5.3.2) to the nearest 0,1 mg in 20 ml vials with crimp cap (6.1) and adding refined oil (5.3.3) to the nearest 0,1 mg in accordance with Table 1.

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 Table 1 — Amounts of PAH standard solutions in oil and refined oil to be used

Calibration curve sample µg/kg	Weighed amount of PAH standard solutions in oil mg	Total mass after adding refined oil g
0,1	10,0	12,500 0
0,8	32,0	5,000 0
1,5	60,0	5,000 0
2,1	84,0	5,000 0
2,8	56,0	2,500 0
3,5	70,0	2,500 0

If it is expected that the level of the (light) PAHs in most of the samples to be analysed is greater than 3,5 µg/kg, adjust the range of the calibration curve. However, ranges exceeding 3,5 µg/kg have not been validated.

2) Norit® SA 4PAH and any other Norit® charcoal are 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.

5.4 Eluents for HPLC analysis.

5.4.1 Solvent A: acetonitrile-water (volume fraction acetonitrile 85 %, water 15 %). Mix 663 g of acetonitrile (5.1.1) and 150 g of water (5.1.5).

5.4.2 Solvent B: acetonitrile (5.1.1).

5.4.3 Solvents C/E: ethyl acetate-acetonitrile (volume fraction ethyl acetate 70 %, acetonitrile 30 %). Mix 630 g of ethyl acetate (5.1.2) and 234 g of acetonitrile (5.1.1).

5.4.4 Solvent D: 2-propanol (5.1.3).

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 HPLC vials with crimp caps, suitable for an autosampler.

6.2 Syringes, capacities: 10 µl; 250 µl.

6.3 Filters³⁾, 0,45 µm.

6.4 Disposable syringes for single use, 5 ml.

6.5 HPLC system, preferably with a heated autosampler.

For the analyses of palm fats, coconut fats or hardened fats, which are prepared in accordance with 8.1.2, a heated sampler is recommended. If no heated autosampler is available, inject the sample preparation immediately, as specified in 8.1.2.

NOTE 1 An example of the individual parts of an HPLC system is given in Annex A. The tubing connections of the HPLC system are given in Annex E⁴⁾.

NOTE 2 An example of the operating conditions of the individual parts of an HPLC system is given in Annexes B to D.

6.6 Chromatography data processing system.

6.7 One-mark volumetric flask with stopper, capacity 50 ml, ISO 1042^[1] class A.

7 Sampling and preparation of the test sample

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^[2].

Prepare the test sample in accordance with ISO 661.

3) Dynagard DG 4P/110/200 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.

4) Suitable systems are commercially available from Dionex (<http://www.dionex.com>), Separations Analytical Instruments (<http://www.separations.nl>), Spark (<http://www.sparkholland.com>) and VWR-Hitachi (<http://www.vwr.com>). This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of products so supplied. Products from other sources may be used if they can be shown to lead to the same results.

8 Sample preparation

8.1 Standard calibration samples.

8.1.1 Liquid oils.

Shake the standard calibration sample to homogenize it completely. Open the cap of the vial and transfer the standard calibration sample to a disposable single use syringe (6.4) equipped with a 0,45 µm filter (6.3). Filter the standard calibration sample into another vial (6.1) and close the vial with a crimp cap. Prepare three extra standard calibration samples of 1,5 µg/kg. These standard calibration samples are analysed first to equilibrate the system.

8.1.2 Palm oil, coconut oil, and hardened fats.

To prevent crystallization, dilute coconut oil, using a dilution factor of 1 as minimum, with the blank sunflower oil (5.3.3). Dilute palm oil, using a dilution factor of 5, with the blank sunflower oil (5.3.3). The dilution factor for hardened fats depends on the iodine value of the fat (degree of hardening).

Heat the palm oil, coconut oil or hardened fat as follows.

Preheat the closed vial with coconut or palm oil at about 60 °C in a heated water bath or drying oven for about 20 min. Shake every few minutes to homogenize the oil.

Dilute hardened fats with warm sunflower oil (5.3.3), using a dilution factor between 1 and 5. If the once-diluted fat is still crystallized, use a greater ratio of sunflower oil. Carry out a pre-test for hardened oils to find the optimal dilution factor.

Weigh, to the nearest 0,000 1 g, an amount of oil corresponding to 1 ml of the warm fat mixture into a vial (6.1).

NOTE The relative densities of various oils are given in Table 2.
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Add 125 µl of 2-propanol (5.1.3) with a syringe (6.2) and close the vial with a crimp cap. Shake the standard calibration sample to homogenize it. Open the cap of the vial and transfer the standard calibration sample to a single use disposable syringe (6.4) equipped with a 0,45 µm filter (6.3).

Filter the standard calibration sample into another vial (6.1) and close the vial with a crimp cap. If crystallization is noticed, heat the vial with the standard calibration sample until it is melted again (see 6.5).

IMPORTANT — If no heated autosampler is available, inject the liquid sample immediately. The vial should not rest in the sampler.

Prepare three extra standard calibration samples of 1,5 µg/kg. These standard calibration samples are analysed first to equilibrate the system.

Table 2 — Relative densities of different types of oil

Type of oil	Mass of 1 ml mg	Relative density
Olive (OV)	914	0,914
Coconut (CN)	923	0,923
Soybean (BO)	916	0,916
Sunflower (SF)	914	0,914
Rapeseed (RP)	913	0,913
Palm kernel (PK)	918	0,918