INTERNATIONAL STANDARD

ISO 22959

First edition
2009-05-15

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

Reference number
ISO 22959:2009(E)

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

Contents

Page

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*.

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 µg/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.

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 $w[C_2H_3N] > 99{,}9$ %.

5.1.2 Ethyl acetate, HPLC grade, mass fraction $w[C_4H_8O_2] > 99{,}8$ %.

5.1.3 2-Propanol, HPLC grade, mass fraction $w[C_3H_8O] > 99{,}9$ %.

5.1.4 Toluene, HPLC grade, mass fraction $w[C_7H_8] > 99{,}9$ %.

5.1.5 Water, HPLC grade.

5.2 Standards.[1)]

5.2.1 Anthracene, mass fraction $w[C_{14}H_{10}] > 99$ %.

5.2.2 Phenanthrene, mass fraction $w[C_{14}H_{10}] > 99$ %.

5.2.3 Fluoranthene, mass fraction $w[C_{16}H_{10}] > 99$ %.

5.2.4 Pyrene, mass fraction $w[C_{16}H_{10}] > 99$ %.

5.2.5 Chrysene, mass fraction $w[C_{18}H_{12}] > 99$ %.

5.2.6 Benzo[*a*]anthracene (1,2-Benzoanthracene), mass fraction $w[C_{18}H_{12}] > 99$ %.

5.2.7 Benzo[*e*]pyrene, mass fraction $w[C_{20}H_{12}] > 99$ %.

5.2.8 Benzo[*a*]pyrene (1,2-Benzopyrene), mass fraction $w[C_{20}H_{12}] > 99$ %.

5.2.9 Perylene, mass fraction $w[C_{20}H_{12}] > 99$ %.

5.2.10 Benzo[*ghi*]perylene (1,12-Benzoperylene), mass fraction $w[C_{22}H_{12}] > 99$ %.

5.2.11 Anthanthrene, mass fraction $w[C_{22}H_{12}] > 99$ %.

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

5.2.13 Coronene, mass fraction $w[C_{24}H_{12}] > 99$ %.

5.2.14 Indeno[1,2,3-*cd*]pyrene, mass fraction $w[C_{22}H_{12}] > 99$ %.

5.2.15 Benzo[*a*]fluoranthene, mass fraction $w[C_{20}H_{12}] > 99$ %.

5.2.16 Benzo[*b*]fluoranthene, mass fraction $w[C_{20}H_{12}] > 99$ %.

5.2.17 Benzo[*k*]fluoranthene, mass fraction $w[C_{20}H_{12}] > 99$ %.

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 charcoal [2]. 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.

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.

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 Filters [3], 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 E [4].

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.

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

8.2 Test portions.

Prepare test portions from the fats and oils test samples using the procedures specified for standard calibration samples in 8.1.1 or 8.1.2, depending on the type of fat. Dilute test samples with PAH concentrations greater than 3,5 µg/kg and analyse a second time.

Prepare diluted test portions by mixing the appropriate mass of test sample with a blank oil (5.3.3) of the same type to the total mass corresponding to 1 ml.

If it is expected that the level of the (light) PAHs in most of the test 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.

9 Procedure

9.1 HPLC analysis

Create a sequence file with the chromatography data processing system (6.6). Place the standard calibration samples and test portions in the autosampler and start the HPLC system. The sequence shall be:

a) 2-propanol (5.1.3) — the chromatogram should be free of relevant peaks, spikes, drift or noise;

b) three extra standard calibration samples to stabilize the system;

c) standard calibration samples (8.1);

d) test portions of oils and fats (8.2);

e) if necessary, standard calibration samples resulting from dilutions (8.1.2).

9.2 Identification of PAHs

Identify the PAHs present in the chromatograms by their retention times. An example of a chromatogram for a standard calibration sample is given in Annex F.

If there is doubt about the identity of a peak, analyse the test sample again. During that analysis, record the excitation and emission spectra of the peak of interest. These spectra can be compared with the model spectra of the PAHs. If the wavelengths applied (Annex C) cannot be used, analyse the test portion(s) and standard calibration samples at different wavelengths.

10 Calculation of individual PAHs

The mass fraction of the individual PAHs, w_{PAH}, is calculated using an external calibration. For this reason, a linear regression curve

$$A_{\mathrm{PAH}} = a w_{\mathrm{PAH}} + b \tag{1}$$

is fitted for each individual PAH. Equation (1) can be rearranged to give the mass fraction of each individual PAH, w_{PAH}, in micrograms per kilogram:

$$w_{\mathrm{PAH}} = \frac{A_{\mathrm{PAH}} - b}{a} \tag{2}$$

where:

A_{PAH} is the peak area of an individual PAH;

w_{PAH} is the PAH content, in micrograms per kilogram, of the sample from the calibration curve;

a is the slope of the calibration curve,

b is the intercept of the calibration curve.

The mass fraction of the individual PAHs, w_{PAH}, is expressed in micrograms per kilogram to one place of decimals.

11 Method validation data

11.1 Accuracy

BCR certified reference material 458 (5.2.18) has been analysed six times. The results are listed in Table 3.

Table 3 — Analysis results for BCR certified reference material 458

PAH	Mean of six analyses µg/kg	Content given by BCR µg/kg
Pyrene	10,02	9,4 ± 1,5
Chrysene	5,00	4,9 ± 0,4
Benzo[*k*]fluoranthene	2,00	1,87 ± 0,18
Benzo[*a*]pyrene	0,99	0,93 ± 0,09
Benzo[*ghi*]perylene	0,98	0,97 ± 0,07
Indeno[1,2,3-*cd*]pyrene	0,99	1,00 ± 0,07

11.2 Within-laboratory precision

The within-laboratory reproducibility has been determined for each individual PAH in four matrices: olive oil, coconut oil, soybean oil and sunflower seed oil. The results are summarized in Annexes G to J.

11.3 Recovery

The recoveries of the PAHs have not been studied. The calibration curve samples and the oil samples are subjected to the same treatment.

11.4 Dynamic range

The concentration range of the calibration curves is 0,1 µg/kg to 3,5 µg/kg.

11.5 Limit of quantification

The limit of quantification of the method is 0,1 µg/kg.

NOTE The limit of quantification of the individual PAHs is < 0,1 µg/kg in the four oils studied.

12 Precision

12.1 International collaborative trial

An interlaboratory test, organized in 2005 to 2006 by FEDIOL/CSL, in which 16 laboratories from seven countries participated, gave the statistical results, evaluated in accordance with ISO 5725-1 [3] and ISO 5725-2 [4], shown in Table K.1.

The values for repeatability and reproducibility limits are expressed for a 95 % probability level and may not be applicable to concentration ranges and matrices other than those given.

12.2 Repeatability

The absolute difference between two independent single test results, obtained with 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 exceed the value of the repeatability limit, r, given in Table K.1.

12.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories by different operators using different equipment, will in not more than 5 % of the cases exceed the value of the reproducibility limit, R, given in Table K.1.

13 Test report

The test report shall contain at least the following information:

a) all information necessary for the complete identification of the sample;

b) a reference to this International Standard;

c) all operating details not specified in this method, or regarded as optional, as well as any incidents which may have influenced the results;

d) the results and the units in which the results are expressed.

Annex A
(informative)

Example of the individual parts of an HPLC system

Table A.1 — HPLC system components

Part	Model [a]	Supplier [a]
On-line degasser (2)	GT-103	Separations
Ternary pump	480	Separations
Autosampler	Triathlon: extra valve syringe 250 µl sample loop 250 µl	Separations
SPE unit		Chrompack
Column thermostat	Mistral	Separations
Fluorescence detector	FP-920	Separations
DACC column	ChromSpher PI 80 × 3 mm	Chrompack
Analytical column	2 Pursuit 5 PAH, 250 × 4,6 mm	Chrompack

[a] Proprietary information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the products or their suppliers. Equivalent products and suppliers may be used if they can be shown to lead to the same results.

Annex B
(informative)

Example of the operating conditions of the pumps of the HPLC system

Table B.1 — Typical HPLC system pump operating conditions

Ternary pump: Separations model 480 [a]								
Eluent (5.4):	A:	Acetonitrile/water						
	B:	Acetonitrile						
	C:	Ethyl acetate/acetonitrile						
Gradient programme (linear steps):								
Time (min)	Flow (µl)	%A	%B	%C		% Water	% Acetonitrile	% Ethyl acetate
0,0	400	100	0	0	→	15	85	0
2,0	400	100	0	0	→	15	85	0
2,5	1 000	100	0	0	→	15	85	0
12,0	1 000	40	60	0	→	6	94	0
20,0	1 000	30	70	0	→	4,5	95,5	0
30,0	1 000	30	70	0	→	4,5	95,5	0
51,0	1 000	0	0	100	→	0	30	70
70,3	1 000	0	0	100	→	0	30	70
71,0	1 500	0	100	0				
78,0	1 500	0	100	0				
78,5	1 500	100	0	0				
86,0	1 500	100	0	0				
86,1	400	100	0	0				
110,0	400	100	0	0				
110,1	0	100	0	0				
Linked event programme:								
Time (min)	G	M	P					
0,0	0	0	0	No action				
8,0	1	3	4	FP-920, perform autozero				
8,5	1	4	4	FP-920, start programme				
8,6	1	2	4	Start data acquisition interface, Turbochrom 4				
78,0	1	3	4	FP-920, autozero				
SPE unit: Chrompack [a]								
Eluent (5.4):	D:	2-Propanol, DACC eluent						
	E:	Ethyl acetate/acetonitrile, flushing eluent						
Flow: 0,35 ml/min								

[a] Proprietary information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the products concerned. Equivalent products may be used if they can be shown to lead to the same results.

Annex C
(informative)

Example of the operating conditions of the column thermostat and the detector of the HPLC system

Table C.1 — Typical HPLC column thermostat and detector operating conditions

Column thermostat: Mistral Separations [a]

Temperature: 20,0 °C

Fluorescence detector: Separations FP-920 [a]

LC programme:

Time	Ex	Em	Gain	PAH detected
min	nm	nm		
0,0	280	400	100	Phenanthrene
6,8			1 000	
6,8	353	420		Anthracene
8,1	350	500		Fluoranthene
9,8			100	
9,8	266	410		Pyrene
13,0	280	420		1,2-Benzoanthracene
17,5	261	400		Chrysene
21,0			1 000	
21,0	240	530		Benzo[*a*]fluoranthene
24,0	324	392		Benzo[*e*]pyrene
26,0	346	438		Benzo[*b*]fluoranthene
				Perylene
31,0			100	
31,0	396	430		Benzo[*k*]fluoranthene
34,1	378	403		Benzo[*a*]pyrene
37,4			1 000	
37,4	290	440		1,2,5,6-Dibenzoanthracene
39,6			100	
				1,12-Benzoperylene
1 000			1 000	
41,6	296	500		Indeno[1,2,3-*cd*]pyrene
44,5			100	
44,5	298	438		Anthanthrene
				Coronene
52,0	280	400		End programme

NOTE The optimal wavelengths of a number of PAHs could not be used because the chromatograms of oil samples often show interfering compounds at those wavelengths.

[a] Proprietary information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the products concerned. Equivalent products may be used if they can be shown to lead to the same results.

Annex D
(informative)

Example of the autosampler programme of the HPLC system

Table D.1 — Typical HPLC column thermostat and detector operating conditions

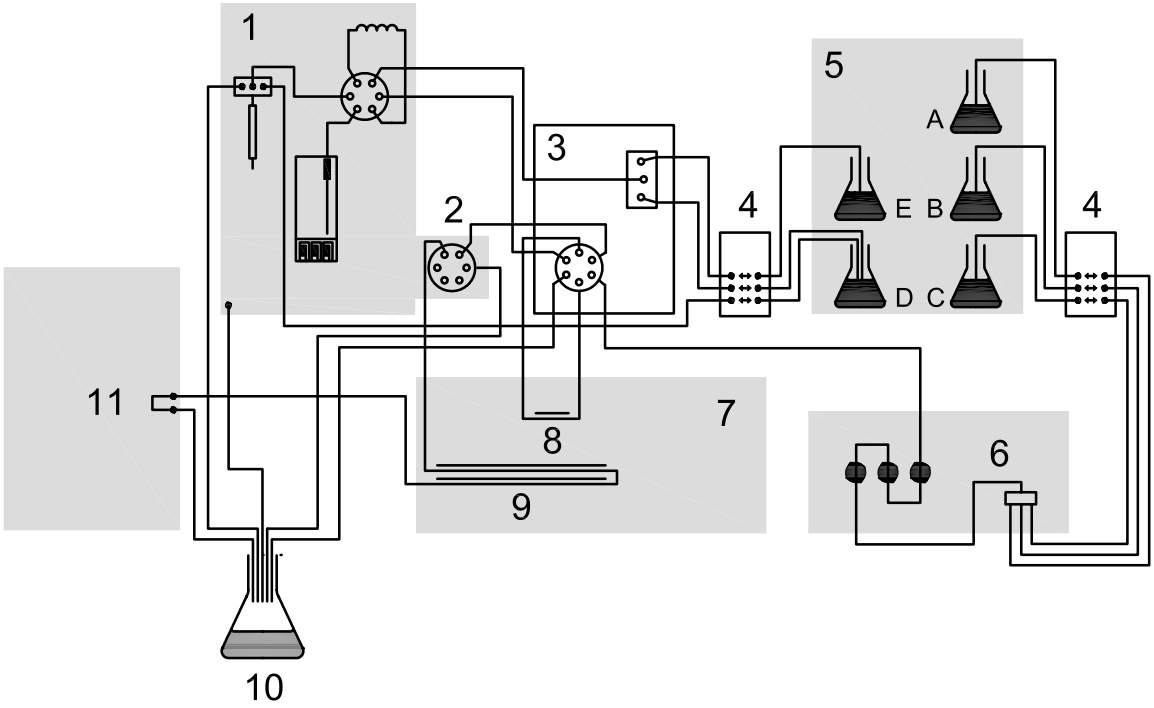
TRIATHLON AUTOSAMPLER USER-PROGRAMME		
STEP	**PROGRAMME LINE**	**EXPLANATION**
001	INJECTOR VALVE POSITION: LOAD	Move injector to load position
002	NEEDLE-WASH VOLUME OF 1 000 µl	Wash needle with 1 000 µl
003	ASPIRATE 0 005 µl AIR SPEED: 1 H: -- mm	Aspirate 5 µl air
004	COMPRESSOR: ON	Provide headspace pressure in sample vial
005	ASPIRATE 0 245 µl SAMPLE SPEED: 1 H: 02 mm	Aspirate 245 µl from sample vial
006	SYRINGE VALVE POSITION: WASTE	Move syringe valve to waste position
007	UNLOAD SYRINGE VOLUME: 0 250 SPEED: 3	Dispense 250 µl from syringe to waste
008	SYRINGE VALVE POSITION: NEEDLE	Move syringe valve to needle position (to continue aspiration of sample)
009	ASPIRATE 0 155 µl SAMPLE SPEED: 1 H: 02 mm	Aspirate 155 µl from sample vial
010	WAIT 0:00:30	Wait 30 s (for pressure to equalize)
011	INJECTOR VALVE POSITION: INJECT	Move injector to inject position (injection on Pi [b] column)
012	ASPIRATE 0 000 µl SAMPLE SPEED: 1 H: 02 mm	Aspirate 0 µl from sample vial (necessary to hold needle in sample vial)
013	COMPRESSOR: OFF	Stop headspace pressure in sample vial
014	NEEDLE-WASH VOLUME OF 1 500 µl	Wash needle with 1 500 µl
015	WAIT : : [a]	Wait (to complete clean up time) Total clean up time — Time step 15 CN — 8,30 min 00:06:40 PK — 8,30 min 00:06:40 OV — 10,00 min 00:08:10 SF — 10,00 min 00:08:10 BO — 10,00 min 00:08:10 RP — 10,00 min 00:08:10
016	ISS-B VALVE POSITION: 6-1	Move extra valve (to waste)
017	WAIT 0:00:05	Wait 5 s (to complete requested clean up time)
018	AUXILIARY PORT 1 ON	Activate auxiliary port 1 (start HPLC gradient)
019	WAIT 0:00:01	Wait 1 s
020	AUXILIARY PORT 1 OFF	Deactivate auxiliary port 1
021	AUXILIARY PORT 2 ON	Activate auxiliary port 2 (Pi column in backflush mode to waste)
022	AUXILIARY PORT 3 ON	Activate auxiliary port 3 (elute washing eluent through SPE unit)
023	WAIT 0:02:25	Wait (to complete elution time of backflush mode to waste)
024	ISS-B VALVE POSITION: 1-2	Move extra valve (to analytical column)
025	WAIT 0:05:00	Wait 5 min (to complete backflush time)
026	AUXILIARY PORT 2 OFF	Deactivate auxiliary port 2 (Pi column in normal mode, eluted with washing eluent)
027	WAIT 0:20:00	Wait 20 min (to complete washing time of Pi column)
028	AUXILIARY PORT 3 OFF	Deactivate auxiliary port 3 (elute clean up eluent through Pi column)
029	WAIT 0:47:00	Wait 47 min (to complete total analysis time)
030	END OF USER-PROGRAMME	End of programme

[a] The total clean up time (total time of steps 12-21) varies depending on the type of oil.

[b] Proprietary information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of the products concerned. Equivalent products may be used if they can be shown to lead to the same results.

Annex E
(informative)

Tubing connections of the HPLC system



Key

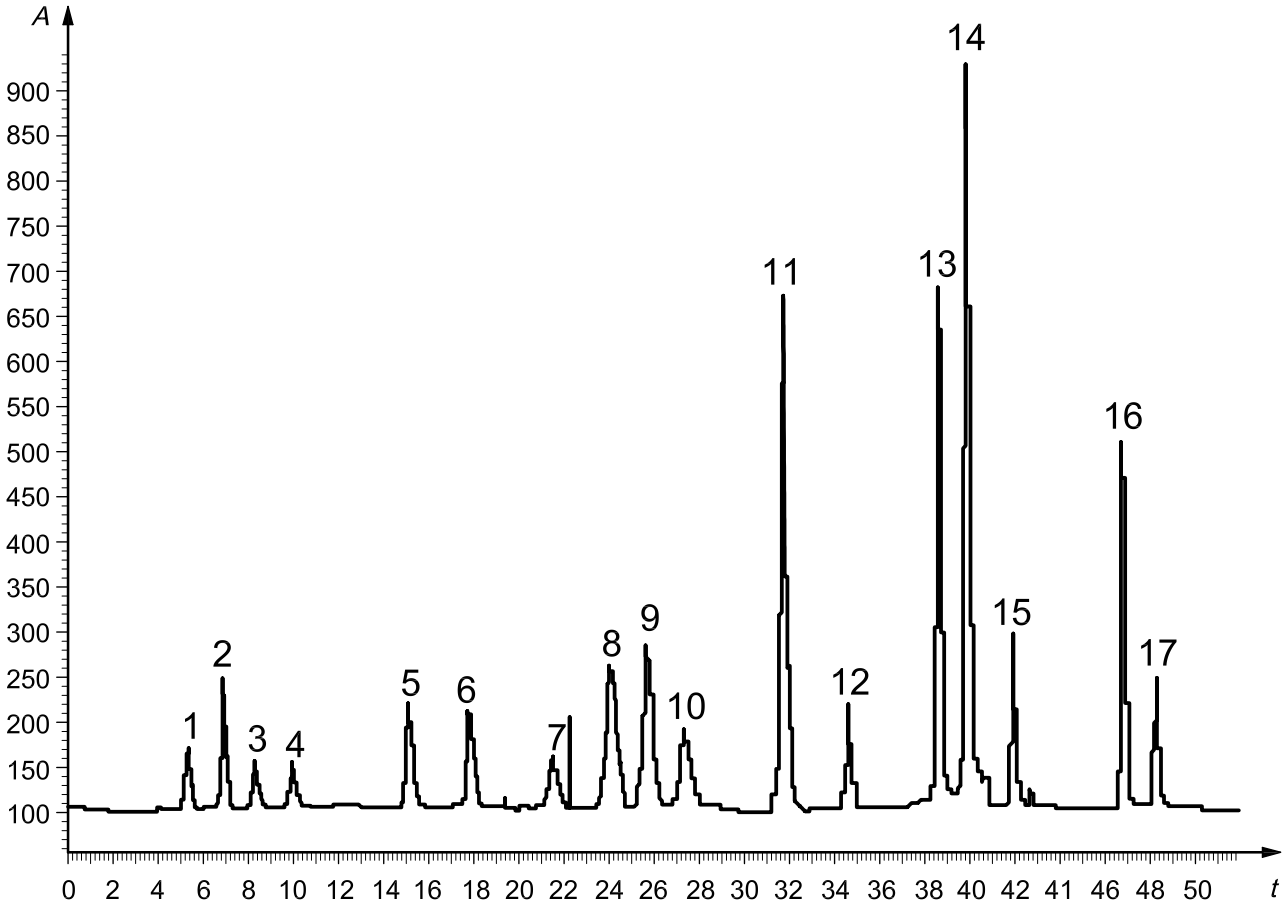
1 autosampler
2 extra valve
3 SPE unit
4 degasser
5 solvent cabinet
6 HPLC pump
7 column thermostat
8 DACC column
9 analytical column
10 waste
11 fluorescence detector

A acetonitrile + water 85 + 15 mobile phase
B acetonitrile mobile phase
C/E acetonitrile + ethyl acetate 30 + 70 mobile phase
D 2-propanol mobile phase

Figure E.1 — Tubing connections

Annex F
(informative)

Chromatogram of a standard calibration sample



Key

1	phenanthrene	11	benzo[*k*]fluoranthene
2	anthracene	12	benzo[*a*]pyrene
3	fluoranthene	13	dibenzo[*a*,*h*]anthracene
4	pyrene	14	benzo[*ghi*]perylene
5	benzo[*a*]anthracene	15	indeno[1,2,3-*cd*]pyrene
6	chrysene	16	anthanthrene
7	benzo[*a*]fluoranthene	17	coronene
8	benzo[*a*]pyrene		
9	benzo[*b*]fluoranthene	*A*	absorbance, arbitrary units
10	perylene	*t*	time, min

Figure F.1 — Standard calibration sample chromatogram

Annex G
(informative)

Determination precision for a sunflower oil, range 0,1 µg/kg to 3,5 µg/kg

Table G.1 — Precision of sunflower oil determination

PAH	Within-laboratory reproducibility		
	$\sqrt{s_{R\,w}}$	$\sqrt{(R_w - \text{value})}$	$\sqrt{(\text{CI})}$ [a]
Phenanthrene [b]	0,027 7	0,078 3	0,055 9
Anthracene	0,011 3	0,032 0	0,022 7
Fluoranthene	0,017 5	0,049 5	0,035 1
Pyrene	0,007 7	0,021 8	0,015 5
Benzo[*a*]anthracene	0,009 6	0,027 2	0,019 2
Chrysene	0,010 3	0,029 1	0,020 8
Benzo[*a*]fluoranthene	0,014 1	0,039 9	0,028 6
Benzo[*e*]pyrene	0,010 1	0,028 6	0,020 4
Perylene	0,015 4	0,043 6	0,030 9
Benzo[*k*]fluoranthene	0,008 5	0,024 0	0,017 1
Benzo[*a*]pyrene [c]	0,008 4	0,023 8	0,016 9
Dibenzo[*a,h*]anthracene	0,013 5	0,038 2	0,023 3
Benzo[*ghi*]perylene	0,008 8	0,024 9	0,017 6
Indeno[1,2,3-*cd*]pyrene	0,009 4	0,026 6	0,018 8
Anthanthrene	—	—	—
Coronene	0,007 8	0,022 1	0,015 6

[a] 95 % confidence interval for the range 0,1 µg/kg to 3,5 µg/kg.

[b] If the estimated level of phenanthrene in sunflower oil is 3,0 µg/kg, then the 95 % confidence intervals are

$$(\sqrt{3,0} + 0,055\,9)^2 = 3,20\ \mu g/kg$$

$$(\sqrt{3,0} - 0,055\,9)^2 = 2,81\ \mu g/kg$$

[c] If the estimated level of benzo[*a*]pyrene in sunflower oil is 0,5 µg/kg, then the 95 % confidence intervals are

$$(\sqrt{0,5} + 0,016\,9)^2 = 0,52\ \mu g/kg$$

$$(\sqrt{0,5} - 0,016\,9)^2 = 0,48\ \mu g/kg$$

Annex H
(informative)

Determination precision for an olive oil, range 0,1 µg/kg to 3,5 µg/kg

Table H.1 — Precision of olive oil determination

PAH	Within-laboratory reproducibility		
	$\sqrt{s_{R\,w}}$	$\sqrt{(R_w - \text{value})}$	$\sqrt{(\text{CI})}$ [a]
Phenanthrene	0,015 1	0,042 7	0,030 4
Anthracene [b]	0,008 8	0,024 9	0,017 7
Fluoranthene	0,018 0	0,050 9	0,036 0
Pyrene	0,010 7	0,030 3	0,021 5
Benzo[*a*]anthracene	0,009 1	0,025 7	0,018 3
Chrysene	0,006 8	0,019 2	0,013 6
Benzo[*a*]fluoranthene	0,013 5	0,038 2	0,027 5
Benzo[*e*]pyrene	0,013 5	0,038 2	0,027 3
Perylene	0,018 3	0,051 8	0,036 6
Benzo[*k*]fluoranthene	0,007 1	0,020 1	0,014 3
Benzo[*a*]pyrene	0,007 6	0,021 5	0,015 2
Dibenzo[*a*,*h*]anthracene [c]	0,009 1	0,025 7	0,018 3
Benzo[*ghi*]perylene	0,007 4	0,020 9	0,014 8
Indeno[1,2,3-*cd*]pyrene	0,007 1	0,020 1	0,014 2
Anthanthrene	—	—	—
Coronene	0,006 2	0,017 5	0,012 4

[a] 95 % confidence interval for the range 0,1 µg/kg to 3,5 µg/kg.

[b] If the estimated level of anthracene in olive oil is 1,0 µg/kg, then the 95 % confidence intervals are

$$(\sqrt{1{,}0} + 0{,}017\,7)^2 = 1{,}04 \text{ µg/kg}$$

$$(\sqrt{1{,}0} - 0{,}017\,7)^2 = 0{,}96 \text{ µg/kg}$$

[c] If the estimated level of dibenzo[*a,h*]anthracene in olive oil is 0,2 µg/kg, then the 95 % confidence intervals are

$$(\sqrt{0{,}2} + 0{,}018\,3)^2 = 0{,}22 \text{ µg/kg}$$

$$(\sqrt{0{,}2} - 0{,}018\,3)^2 = 0{,}18 \text{ µg/kg}$$

Annex I
(informative)

Determination precision for a soybean oil, range 0,1 µg/kg to 3,5 µg/kg

Table I.1 — Precision of soybean oil determination

PAH	Within-laboratory reproducibility		
	$\sqrt{s_{R\,w}}$	$\sqrt{(R_w - \text{value})}$	$\sqrt{(\text{CI})}$ [a]
Phenanthrene	0,029 6	0,083 7	0,059 3
Anthracene	0,010 7	0,030 3	0,021 4
Fluoranthene	0,017 8	0,050 3	0,035 7
Pyrene [b]	0,009 6	0,027 2	0,019 2
Benzo[*a*]anthracene	0,012 4	0,035 1	0,024 8
Chrysene	0,011 9	0,033 6	0,023 8
Benzo[*a*]fluoranthene	0,019 8	0,056 0	0,040 2
Benzo[*e*]pyrene	0,010 7	0,030 3	0,020 8
Perylene	0,021 4	0,060 5	0,042 9
Benzo[*k*]fluoranthene	0,011 0	0,031 1	0,022 1
Benzo[*a*]pyrene	0,009 5	0,026 9	0,019 0
Dibenzo[*a,h*]anthracene	0,010 4	0,029 4	0,021 0
Benzo[*ghi*]perylene [c]	0,008 9	0,025 2	0,017 8
Indeno[1,2,3-*cd*]pyrene	0,011 5	0,032 5	0,023 1
Anthanthrene	—	—	—
Coronene	0,007 3	0,020 6	0,014 6

[a] 95 % confidence interval for the range 0,1 µg/kg to 3,5 µg/kg.

[b] If the estimated level of pyrene in soybean oil is 2,0 µg/kg, then the 95 % confidence intervals are

$$(\sqrt{2,0} + 0,019\,2)^2 = 2,05\ \mu g/kg$$

$$(\sqrt{2,0} - 0,019\,2)^2 = 1,95\ \mu g/kg$$

[c] If the estimated level of benzo[*ghi*]perylene in soybean oil is 0,1 µg/kg, then the 95 % confidence intervals are

$$(\sqrt{0,1} + 0,017\,8)^2 = 0,11\ \mu g/kg$$

$$(\sqrt{0,1} - 0,017\,8)^2 = 0,09\ \mu g/kg$$

Annex J
(informative)

Determination precision for a coconut oil, range 0,1 µg/kg to 3,5 µg/kg

Table J.1 — Precision of coconut oil determination

PAH	Within-laboratory reproducibility		
	$\sqrt{s_{R\,w}}$	$\sqrt{(R_w - \text{value})}$	$\sqrt{(\text{CI})}$ [a]
Phenanthrene	0,019 0	0,053 7	0,038 0
Anthracene	0,011 2	0,031 7	0,022 5
Fluoranthene	0,017 2	0,048 5	0,034 4
Pyrene	0,011 0	0,031 1	0,022 2
Benzo[*a*]anthracene [b]	0,012 0	0,033 9	0,024 0
Chrysene	0,013 5	0,038 2	0,027 2
Benzo[*a*]fluoranthene	0,016 0	0,045 2	0,033 0
Benzo[*e*]pyrene	0,014 7	0,041 6	0,029 6
Perylene	0,023 0	0,065 2	0,046 2
Benzo[*k*]fluoranthene	0,009 0	0,025 4	0,018 1
Benzo[*a*]pyrene	0,007 9	0,022 0	0,015 6
Dibenzo[*a*,*h*]anthracene	0,014 6	0,041 3	0,029 3
Benzo[*ghi*]perylene	0,011 6	0,032 8	0,023 4
Indeno[1,2,3-*cd*]pyrene	0,010 1	0,028 6	0,020 3
Anthanthrene	0,013 6	0,038 5	0,027 5
Coronene [c]	0,007 0	0,020 0	0,014 1

[a] 95 % confidence interval for the range 0,1 µg/kg to 3,5 µg/kg.

[b] If the estimated level of benzo[*a*]anthracene in coconut oil is 3,5 µg/kg, then the 95 % confidence intervals are

$$(\sqrt{3,5} + 0,024\,0)^2 = 3,59\ \mu g/kg$$

$$(\sqrt{3,5} - 0,024\,0)^2 = 3,41\ \mu g/kg$$

[c] If the estimated level of coronene in coconut oil is 0,3 µg/kg, then the 95 % confidence intervals are

$$(\sqrt{0,3} + 0,014\,1)^2 = 0,32\ \mu g/kg$$

$$(\sqrt{0,3} - 0,014\,1)^2 = 0,28\ \mu g/kg$$

Annex K
(informative)

Interlaboratory test

The interlaboratory test at the international level in 2005/2006 by FEDIOL/CSL, in which 16 laboratories participated, each obtaining two test results for each sample (blind duplicate), gave the statistical results (evaluated in accordance with ISO 5725-2:1994 [4]) summarized in Table K.1.

Table K.1 — Results of the interlaboratory test

PAH	No. labs [a]	Mean	Standard deviation of repeatability	Coefficient of variation of repeatability	Repeatability limit	Standard deviation of reproducibility	Coefficient of variation of reproducibility	Reproducibility limit
			s_r	CV(r)	r = 2,8 s_r	s_R	CV(R)	R = 2,8 s_R
		µg/kg	µg/kg	%		µg/kg	%	
Coconut oil 1								
Benzo[*a*]anthracene	13	0,50	0,07	13,6	0,19	0,10	19,8	0,28
Chrysene	14	0,87	0,12	13,6	0,33	0,19	22,1	0,54
Dibenzo[*a*,*h*]anthracene	12	1,15	0,04	3,1	0,10	0,10	9,1	0,29
Benzo[*b*]fluoranthene	13	0,58	0,05	8,3	0,13	0,10	16,7	0,27
Benzo[*k*]fluoranthene	12	2,95	0,11	3,6	0,30	0,29	9,9	0,82
Benzo[*ghi*]perylene	13	2,85	0,17	6,1	0,48	0,65	22,8	1,82
Benzo[*a*]pyrene	12	2,63	0,07	2,8	0,21	0,27	10,3	0,76
Indeno[1,2,3-*cd*] pyrene	13	2,84	0,23	8,2	0,65	0,79	27,6	2,20
Coconut oil 2								
Benzo[*a*]anthracene	13	3,07	0,08	2,6	0,22	0,26	8,4	0,73
Chrysene	13	2,94	0,03	1,2	0,10	0,54	18,4	1,51
Dibenzo[*a*,*h*]anthracene	14	4,41	0,07	1,5	0,18	0,30	6,8	0,84
Benzo[*b*]fluoranthene	13	2,33	0,06	2,7	0,17	0,19	8,1	0,53
Benzo[*k*]fluoranthene	14	0,57	0,04	6,6	0,11	0,09	15,2	0,24
Benzo[*ghi*]perylene	11	0,67	0,05	8,1	0,15	0,17	25,2	0,47
Benzo[*a*]pyrene	14	0,82	0,03	3,3	0,07	0,03	17,7	0,41
Indeno[1,2,3-*cd*] pyrene	12	0,99	0,10	9,9	0,27	0,10	21,1	0,58

Table K.1 (*continued*)

PAH	No. labs [a]	Mean	Standard deviation of repeatability s_r	Coefficient of variation of repeatability CV(r)	Repeatability limit $r = 2{,}8\ s_r$	Standard deviation of reproducibility s_R	Coefficient of variation of reproducibility CV(R)	Reproducibility limit $R = 2{,}8\ s_R$
		µg/kg	µg/kg	%		µg/kg	%	
Sunflower oil 1								
Benzo[*a*]anthracene	16	1,00	0,08	7,7	0,22	0,13	13,5	0,38
Chrysene	14	0,49	0,05	11,0	0,15	0,13	26,5	0,36
Dibenzo[*a*,*h*]anthracene	16	1,45	0,08	5,2	0,21	0,14	9,4	0,38
Benzo[*b*]fluoranthene	13	0,54	0,03	6,4	0,10	0,07	13,1	0,20
Benzo[*k*]fluoranthene	16	2,44	0,16	6,5	0,44	0,27	11,2	0,77
Benzo[*ghi*]perylene	13	2,20	0,09	4,1	0,25	0,21	9,5	0,59
Benzo[*a*]pyrene	14	2,95	0,11	3,8	0,31	0,28	9,5	0,78
Indeno[1,2,3-*cd*] pyrene	13	2,72	0,21	7,6	0,58	0,49	17,9	1,36
Sunflower oil 2								
Benzo[*a*]anthracene	15	2,38	0,05	2,2	0,15	0,25	10,4	0,69
Chrysene	15	3,03	0,12	3,8	0,32	0,32	15,5	0,89
Dibenzo[*a*,*h*]anthracene	16	5,01	0,12	2,5	0,35	0,41	8,1	1,14
Benzo[*b*]fluoranthene	16	2,62	0,08	3,1	0,22	0,34	12,8	0,94
Benzo[*k*]fluoranthene	15	0,75	0,00	0,0	0,00	0,07	8,9	0,18
Benzo[*ghi*]perylene	15	0,77	0,07	9,4	0,20	0,22	28,9	0,62
Benzo[*a*]pyrene	14	0,87	0,03	3,1	0,07	0,10	10,9	0,27
Indeno[1,2,3-*cd*] pyrene	11	0,96	0,08	8,3	0,22	0,16	16,8	0,45
Palm oil								
Benzo[*a*]anthracene	10	0,53	0,00	0,0	0,00	0,11	20,0	0,30
Chrysene	10	0,91	0,10	10,8	0,27	0,18	19,5	0,49
Dibenzo[*a*,*h*]anthracene	12	0,84	0,04	4,2	0,10	0,14	16,4	0,38
Benzo[*b*]fluoranthene	12	0,85	0,06	6,8	0,16	0,14	16,6	0,39
Benzo[*k*]fluoranthene	12	0,75	0,13	16,9	0,36	0,14	18,8	0,40
Benzo[*ghi*]perylene	10	0,83	0,12	14,1	0,33	0,17	20,3	0,47
Benzo[*a*]pyrene	11	0,78	0,04	5,5	0,12	0,17	21,2	0,46
Indeno[1,2,3-*cd*] pyrene	10	0,95	0,14	15,3	0,41	0,22	23,1	0,62

[a] After eliminating outliers.

Bibliography

[1] ISO 1042, *Laboratory glassware — One-mark volumetric flasks*

[2] ISO 5555, *Animal and vegetable fats and oils — Sampling*

[3] ISO 5725-1:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 1: General principles and definitions*

[4] ISO 5725-2:1994, *Accuracy (trueness and precision) of measurement methods and results — Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method*

[5] VAN STIJN, F., KERKHOFF, M.A.T., VANDEGINSTE, B.G.M. Determination of polycyclic aromatic hydrocarbons in edible oils and fats by on-line donor-acceptor complex chromatography and high-performance liquid chromatography with fluorescence detection, *J. Chromatogr. A* 1996, **750**, pp. 263-273

ICS 67.200.10

Price based on 22 pages