SLOVENSKI STANDARD
SIST-TS CEN/TS 16621:2014

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Analize živil - Določevanje benzo[a]pirena, benz[a]antracena, krizena in benzo[b] fluorantena v živilih s tekočinsko kromatografijo visoke ločljivosti s fluorescentno detekcijo (HPLC-FD)

Food analysis - Determination of benzo[a]pyrene, benz[a]anthracene, chrysene and benzo[b]fluoranthene in foodstuffs by high performance liquid chromatography with fluorescence detection (HPLC-FD)

Lebensmittelanalytik - Bestimmung von Benzo(a)pyren, Benz(a)anthracen, Chrysen und Benzo(b)fluoranthen in Lebensmitteln mittels Hochleistungs-Flüssigkeitschromatographie mit Fluoreszenzdetektion (HPLC-FD)

Analyse des produits alimentaires - Dosage du benzo(a)pyrène, benzo(a)anthracène, chrysène et benzo(b)fluoranthène dans les denrées alimentaires par chromatographie en phase liquide à haute performance avec détection de fluorescence (HPLC-FD)

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67.050	Splošne preskusne in analizne metode za živilske proizvode	General methods of tests and analysis for food products

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English Version

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

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Foreword

This document (CEN/TS 16621:2014) has been prepared by Technical Committee CEN/TC 275 “Food analysis - Horizontal methods”, the secretariat of which is held by DIN.

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1 Scope

This Technical Specification specifies a method for the determination of benzo[*a*]pyrene (BaP) plus benz[*a*]anthracene (BaA), benzo[*b*]fluoranthene (BbF) and chrysene (CHR) in several food matrices. The method is based on size exclusion chromatography (SEC) cleanup, followed by quantification with high performance liquid chromatography (HPLC) with programmable fluorescence detection. This method has been in-house validated via the analysis of spiked samples of edible olive oil, fresh mussels, smoked fish, smoked meat products, processed cereal-based foods for young children, infant formulae, chocolate and food supplements (isoflavones) at levels ranging from 0,25 µg/kg to 1,00 µg/kg and from 4,95 µg/kg to 23,53 µg/kg, depending on the Polycyclic Aromatic Hydrocarbon (PAH) or the matrix. This method complies with the performance characteristics specified for BaP, BaA, BbF and CHR in current legislation [3].

The method has been shown to be applicable to a variety of additional matrices as meat products, fresh fish, paprika, roasted coffee, bread, herbs, breakfast cereals, beer, sunflower oil, olives and fried tomato, with a limit of quantification below 0,5 µg/kg.

In addition, the method was tested in-house and shown to be applicable also for the quantification of the other 12 PAHs of the 15+1 EU priority PAHs set (benzo[*c*]fluorene (BcL), benzo[*j*]fluoranthene (BjF), benzo[*k*]fluoranthene (BkF), cyclopenta[*cd*]pyrene (CPP), dibenz[*a,h*]anthracene (DhA), dibenzo[*a,e*]pyrene (DeP), benzo[*ghi*]perylene (BgP), dibenzo[*a,h*]pyrene (DhP), dibenzo[*a,i*]pyrene (DiP), dibenzo[*a,l*]pyrene (DlP), indeno[1,2,3-*cd*]pyrene (IcP), 5-methylchrysene (5MC)) in all matrices listed above and at similar level ranges, except for CPP, where a UV detection had to be used with limits of quantification above 8 µg/kg.

For the determination of PAHs in edible fats and oils, two other standards are also available, EN ISO 22959 and EN ISO 15753 (see [1] and [2]).

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN ISO 3696:1995, *Water for analytical laboratory use - Specification and test methods (ISO 3696:1987)*

3 Principle

The PAHs are extracted from solid matrices with dichloromethane. In case of edible oils, the samples are simply dispersed in dichloromethane. Aliquots of crude extracts in dichloromethane are purified by SEC. The final extracts are analysed by HPLC under gradient conditions with programmable fluorescence detection.

4 Reagents

Use only reagents of recognized analytical grade and water complying with grade 1 of EN ISO 3696:1995, unless otherwise specified. Solvents shall be of quality for HPLC analysis. For storing and expiring dates of use of substances and commercially available solutions, supplier indications or certificates shall be followed. Refrigerated standard solutions shall reach room temperature before being used.

WARNING 1 — Some PAHs are considered carcinogenic. Persons using this document should be familiar with normal laboratory practices. It is the responsibility of the user of this document to apply practices which are in agreement with applicable occupational safety and health practices.

WARNING 2 — Dispose chemical waste according to applicable environmental rules and regulations.

WARNING 3 — PAHs are degraded by UV light. Protect PAHs solutions from light (keep in the dark, use aluminium foil or amber glassware).

WARNING 4 — The analyst shall ensure that samples do not become contaminated during sample preparation. Containers shall be rinsed with high purity acetone or hexane before use to minimize the risk of contamination. Wherever possible, apparatus and equipment coming into contact with the sample shall be made of inert materials such as aluminium, glass or polished stainless steel. Some precaution is needed when using plastics as polypropylene or PTFE because the analytes may be adsorbed onto these materials.

4.1 Helium purified compressed gas (purity equivalent to 99,995 % or better). For solvent degassing, if needed.

4.2 Nitrogen purified compressed gas (purity equivalent to 99,995 % or better).

4.3 Acetone.

4.4 *n*-Hexane.

4.5 Dichloromethane.

4.6 Acetonitrile.

4.7 Methoxychlor.

4.8 Perylene.

4.9 Sulfur.

4.10 Corn oil, commercial.

4.11 HPLC mobile phase solvent A: Water.

The mobile phase solvent A should be degassed.

4.12 HPLC mobile phase solvent B: Acetonitrile (4.6).

The mobile phase solvent B should be degassed.

4.13 Cyclohexane.

4.14 Ethyl acetate.

4.15 Mixture of cyclohexane and ethyl acetate.

Mix one part per volume of cyclohexane (4.13) with one part per volume of ethyl acetate (4.14).

4.16 Anhydrous sodium sulphate.

4.17 Polycyclic aromatic hydrocarbons.

4.17.1 Benzo[*a*]pyrene (BaP).

4.17.2 Chrysene (CHR).

4.17.3 Benzo[*b*]fluoranthene (BbF).

4.17.4 Benz[*a*]anthracene (BaA).

4.17.5 Benzo[*k*]fluoranthene (BkF).

4.17.6 Dibenzo[*a,h*]anthracene (DhA).

4.17.7 Benzo[*g,h,i*]perylene (BgP).

4.17.8 Indeno[1,2,3-*cd*]pyrene (IcP).

4.17.9 Benzo[*c*]fluorene (BcL).

4.17.10 Cyclopenta[*c,d*]pyrene (CPP).

4.17.11 5 – Methylchrysene (5MC).

4.17.12 Benzo[*j*]fluoranthene (BjF).

4.17.13 Dibenzo[*a,l*]pyrene (DlP).

4.17.14 Dibenzo[*a,e*]pyrene (DeP).

4.17.15 Dibenzo[*a,i*]pyrene (DiP).

4.17.16 Dibenzo[*a,h*]pyrene (DahP).

4.17.17 15+1 EU priority PAHs standard solution containing 10 µg/ml each, in appropriate organic solvent, preferably acetonitrile.

4.18 PAH4 standard solution

Prepare a standard solution of 10 µg/ml PAH4 in acetonitrile by weighing carefully the proper amounts of the 4 PAHs (BaP, BaA, BbF and CHR) individually in acetonitrile. Store this solution under refrigeration conditions. A solution stored in this way is stable for at least 12 months. If longer stability is proven, the solution can still be applied.

4.19 PAH4 stock solution

Prepare a stock solution of 500 ng/ml in acetonitrile by diluting exactly 500 µl of the 10 µg/ml PAH4 standard solution (4.18) to 10 ml with acetonitrile (4.6) into a calibrated 10 ml volumetric flask. Store this solution under refrigeration conditions. A solution stored in this way is stable for at least 12 months. If longer stability is proven, the solution can still be applied.

4.20 PAH4 working solution

Prepare a working solution of 50 ng/ml in acetonitrile, by diluting 1 ml of the 500 ng/ml PAH4 stock solution in acetonitrile (4.19) up to 10 ml with acetonitrile (4.6) into a calibrated 10 ml volumetric flask. Store this solution under refrigeration conditions. A solution stored in this way is stable for at least six months. If longer stability is proven, the solution can still be applied.

4.21 15+1 PAHs stock solution

Prepare a stock solution of 500 ng/ml in acetonitrile by pipetting exactly 500 µl of the 10 µg/ml 15+1 PAHs standard solution (4.17.17) into a calibrated 10 ml volumetric flask. Take them to dryness by evaporation under nitrogen, and redissolve in 10 ml of acetonitrile (4.6). Store this solution under refrigeration conditions. A solution stored in this way is stable for at least 12 months. If longer stability is proven, the solution can still be applied.

In case of commercial availability of 15+1 EU priority PAHs standard solution containing 10 µg/ml each, in acetonitrile, the 15+1 PAHs stock solution can be prepared directly by diluting 500 µl of that solution up to 10 ml with acetonitrile.

4.22 15+1 PAHs working solution

Prepare a working solution of 50 ng/ml in acetonitrile, by diluting 1 ml of the 500 ng/ml 15+1 PAHs stock solution in acetonitrile (4.21) to 10 ml with acetonitrile into a calibrated 10 ml volumetric flask. Store this solution under refrigeration conditions. A solution stored in this way is stable for at least six months. If longer stability is proven, the solution may still be applied.

5 Apparatus

Usual laboratory glassware and equipment and, in particular, the following:

5.1 High speed blender, for solid samples and **vortex mixer** for liquid samples.

5.2 Filter papers, suitable for qualitative analysis, prefolded.

5.3 Round bottomed glass tubes, of 250 ml capacity, suitable for the high speed blender.

5.4 Evaporator, with water bath and flushing nitrogen capability.

5.5 Analytical balance, accuracy to the nearest 0,000 1 g.

5.6 Laboratory balance, accuracy to the nearest 0,1 g.

5.7 Glass syringe, of 5 ml capacity.

5.8 Microsyringes, of 250 µl, 500 µl and 1 000 µl capacity.

5.9 Calibrated volumetric flasks, of 5 ml and 10 ml capacity.

5.10 Displacement pipettes, of 200 µl, with appropriate tips.

5.11 Glass vials, approximately 1,8 ml capacity and crimp caps.

5.12 Graduated pipette, of 5 ml capacity.

5.13 Size-Exclusion Chromatography (SEC) system, comprising the following:

5.13.1 HPLC pump (isocratic), capable of pumping 5 ml/min pulse free.

5.13.2 Injection system, suitable for 1,0 ml and 0,2 ml injection volume.

5.13.3 Two SEC cleanup columns, 19 mm x 150 mm and 19 mm x 300 mm, connected in series, packed with high-performance, fully porous, highly cross-linked, styrene divinylbenzene copolymer particles, 10 nm pore size with nominal particle size of 15 µm.

5.13.4 UV detector, capable to provide λ = 254 nm.

5.13.5 Fraction collector.

5.13.6 Recorder, integrator or computer based data processing system.

5.13.7 Calibration

Connect the columns to the system and calibrate the SEC cleanup columns following the instructions of the manufacturer. The calibration solution requires the following compounds: corn oil, methoxychlor, perylene and sulfur. The system calibration will determine the time spent from the injection of the sample to the elution of PAHs by using perylene as indicator. The fraction or fractions containing the PAHs can be selected by programming the fractions collector.

NOTE Other SEC columns can be used provided that the SEC system, after calibration, is able to include the PAHs in the selected fraction or fractions, with acceptable recovery rates and free of disturbing interferences.

5.14 HPLC apparatus, comprising the following:

5.14.1 Injection system, suitable for 100 µl injection volume.

5.14.2 Mobile phase pump (gradient), capable of pumping 1 ml/min pulse free.

5.14.3 Programmable fluorescence detector.

5.14.4 Computer based data processing system.

5.14.5 Analytical reverse-phase HPLC specific for PAHs, separating column, C18, base deactivated octadecyl silane (ODS) (recommended 4,6 mm x 250 mm column with 5 µm particle size), and a suitable corresponding reverse phase guard column.

5.14.6 Degasser, (optional).

5.14.7 Column oven, capable to operate at 28 °C ± 1 °C.

5.14.8 UV detector, capable to provide λ = 223 nm.

5.15 PTFE filters, 0,20 µm or 0,45 µm.

5.16 Glass tubes, of 10 ml capacity, suitable for the evaporator (5.4) and the fraction collector (5.13.5).

5.17 Glass tubes, of 10 ml capacity, capped.

5.18 Grinding mill.

6 Procedure

6.1 Sample preparation

In general, only the edible parts of foodstuffs will be analysed. The edible parts of shellfish shall be thoroughly washed with water and dried by slightly pressing with filter papers. The edible portions to be analysed shall be ground and well mixed. They can be stored in closed glass or aluminium containers under frozen conditions up to the moment of their analysis.

6.2 Extraction

Weigh, to the nearest 0,1 g, a 25 g test portion of prepared sample into a 250 ml-round bottomed glass tube, (5.3). Add 100 ml of dichloromethane (4.5), (V_1, see Clause 8). Blend for 3 min with a high speed blender (5.1).

Filter an aliquot of the lower organic layer through a prefolded paper filter. The filtered extract shall be clear, otherwise repeat this step with some anhydrous sodium sulphate (4.16) in the filter. Filter an aliquot of the filtered extract through a 0,20 µm or 0,45 µm PTFE filter (5.15), using a 5 ml glass syringe (5.7) and purify by SEC.

In the case of samples of edible oils, the extraction step is replaced by just a dispersion of the sample in dichloromethane as follows: Pour a 5 ml portion of oil in a previously tared glass tube (5.17) and take note of the sample weight to the nearest 0,1 g. Add 5 ml of dichloromethane (4.5), cap the tube and mix once by inversion. After that, mix by stirring one min in vortex mixer (5.1) and filter an aliquot of the mixture through a 0,20 µm or 0,45 µm PTFE filter (5.15).

NOTE 1 The amounts of oil and solvent can be modified depending on the convenience, but maintaining the same rate of 1 + 1 (for example 2 ml oil + 2 ml dichloromethane).

A mixture of 1 part per volume of cyclohexane and 1 part per volume of ethyl acetate (4.15) can be used as an alternative extraction solvent to dichloromethane. If so, it shall also be used for the equilibration, elution and washing of the SEC system (see 6.3).

NOTE 2 A mixture of 1 part per volume of cyclohexane and 1 part per volume of ethyl acetate gives longer elution times in the SEC and lower final recoveries than dichloromethane, although they always fulfil the legal requirements of the EU (see [3]).

6.3 SEC cleanup

Equilibrate the whole SEC system (5.13) previously by passing dichloromethane at a speed of 5 ml/min during approximately 30 min. Inject 0,2 ml (V_2, see Clause 8) of the filtered extract into the SEC system. Elute the PAHs by passing dichloromethane at a flow rate of 5 ml/min. Collect the fraction or fractions corresponding to the elution time of the PAHs according to the elution times established during the calibration of the SEC system. PAHs are eluted typically from 920 s to 1 170 s under the described conditions.

After the elution of the PAHs, equilibrate the SEC system again by passing dichloromethane during no less than 10 min before the next injection.

Evaporate the collected fraction or fractions to dryness in an evaporator under a gentle stream of nitrogen (for example, at 34 kPa (= 5 psi) and 27 °C to 28 °C), and redissolve in 1 ml (V_3, see Clause 8) of acetonitrile (4.6). If the HPLC is not performed immediately, store the final extract in a refrigerator.

7 HPLC analysis

7.1 HPLC operation conditions

When the column specified in (5.14.5) and the mobile phases A and B specified in 4.11 and 4.12 were used the setting as given in Table 1 were found to be appropriate:

Table 1 — Gradient conditions

Time min	**Flowrate** ml/min	**Mobile phase A** %	**Mobile phase B** %
0	1	50	50
3	1	50	50
30	1	0	100
40	1	0	100
43	2	0	100
70	2	0	100
85	1	50	50