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Vegetable oils — Determination of mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH) with online-coupled high performance liquid chromatography-gas chromatography-flame ionization detection (HPLC-GC-FID) analysis — Method for low limit of quantification

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ISO copyright office
CP 401 • Ch. de Blandonnet 8
CH-1214 Vernier, Geneva
Phone: +41 22 749 01 11
Email: copyright@iso.org
Website: www.iso.org

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Foreword

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This document was prepared by Technical Committee ISO/TC 34 *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*, in collaboration with the European Committee for Standardization (CEN) Technical Committee CEN/TC 307, *Oilseeds, vegetable and animal fats and oils and their by-products — Methods of sampling and analysis*, in accordance with the Agreement on technical cooperation between ISO and CEN (Vienna Agreement).

Any feedback or questions on this document should be directed to the user's national standards body. A complete listing of these bodies can be found at www.iso.org/members.html.

Introduction

In order to achieve a low limit of quantification (LOQ), the method contains additional and partially modified processing steps, specifications for the uniform processing of defined product groups and additional requirements for system suitability compared to EN 16995:2017.

The method has been tested in an interlaboratory study via the analysis of both naturally contaminated and spiked vegetable oil samples, ranging from 1 mg/kg to 75 mg/kg for MOSH, and from 1 mg/kg to 7 mg/kg for MOAH.

Vegetable oils — Determination of mineral oil saturated hydrocarbons (MOSH) and mineral oil aromatic hydrocarbons (MOAH) with online-coupled high performance liquid chromatography-gas chromatography-flame ionization detection (HPLC-GC-FID) analysis — Method for low limit of quantification

1 Scope

This document specifies a procedure for the determination of saturated and aromatic hydrocarbons (from C10 to C50) in vegetable fats and oils using the online-coupled high performance liquid chromatography-gas chromatography-flame ionization detection (HPLC-GC-FID).[4][5][6] This document does not apply to other matrices.

The method is applicable for the analysis of mineral oil saturated hydrocarbons (MOSH) and/or mineral oil aromatic hydrocarbons (MOAH).

According to the results of the interlaboratory studies, the method has been proven suitable for MOSH mass concentrations above 3 mg/kg and MOAH mass concentrations above 2 mg/kg.

In case of suspected interferences, the fossil origin of the MOSH and MOAH fraction can be verified by examination by GC×GC-MS.

An alternative method for the epoxidation of the MOAH fraction (performic acid epoxidation) is proposed in Annex C. This alternative method provides comparable results to the ethanolic epoxidation of the MOAH fraction described in 8.6. This alternative method for epoxidation has proven to be efficient for samples with a high amount of interferences in the MOAH fraction (e.g. tropical oils).[14]

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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.

ISO and IEC maintain terminology databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at https://www.electropedia.org/

3.1
mineral oil saturated hydrocarbons
MOSH
paraffinic (open-chain, usually branched) and naphthenic (cyclic, alkylated) hydrocarbons in the boiling range of *n*-alkanes with a chain length of 10 to 50 carbon atoms, which are obtained from mineral oil by this method by means of online-coupled high performance liquid chromatography-gas chromatography-flame ionization detection (HPLC-GC-FID)

3.2
mineral oil aromatic hydrocarbons
MOAH
aromatic mainly alkylated hydrocarbons from mineral oil in the boiling range of *n*-alkanes with a chain length of 10 to 50 carbon atoms, determined by means of online-coupled high performance liquid chromatography-gas chromatography-flame ionization detection (HPLC-GC-FID)

3.3
unresolved complex mixture
UCM
complex mixture of saturated or aromatic hydrocarbons not resolved by gas chromatography such as branched paraffins, alkylated naphthenes and alkylated aromatics, that produces a hump when analysed by gas chromatography-flame ionization detection (GC-FID)

3.4
polyolefin oligomeric saturated hydrocarbons
POSH
synthetic hydrocarbons from oligomers of polyolefins, such as polyethylene, polypropylene and polybutylenes

Note 1 to entry: Food contact uses comprise plastic bags, containers or films, heat sealable layers and other lamination as well as adhesives and plasticizers.

Note 2 to entry: POSH can be distinguished from mineral oil saturated hydrocarbons (MOSH) by their chromatographic pattern, but it is difficult to differentiate and chromatographically separate them from the MOSH if both are present.[5]

3.5
resin oligomeric saturated hydrocarbons
ROSH

synthetic saturated hydrocarbons (oligomers from monoterpenes, cyclopentadienes and other C5- or C9-monomeres) that are ingredients of hot-melt adhesives and can migrate into the sample mostly via gas phase transfer or via direct contact

3.6
resin oligomeric aromatic hydrocarbons
ROAH
synthetic aromatic hydrocarbons that are ingredients of hot-melt adhesives and can migrate into the sample mostly via gas phase transfer or by direct contact

3.7
poly-alpha-olefins
PAO
synthetic iso-paraffins with short and long side chains, used as lubricants or in adhesives and hotmelts

Note 1 to entry: When analysed by gas chromatography-flame ionization detection (GC-FID), they are recognized by series of rather narrow humps of unresolved branched hydrocarbons with regular distance between them.[5]

4 Principle

The sample is saponified and from the unsaponifiable residue, purified fractions are obtained following additional steps. These fractions are separated on a silica gel column of the HPLC-GC-FID system into MOSH

and MOAH fractions; each is transferred separately to the GC by online coupling. Most of the solvent is removed via a solvent vapour exit between the uncoated pre-column and the GC separation column.

In order to meet the requirements of the various interfering accompanying substances occurring in the samples, specific sample preparation procedures are described for different product groups. However, epoxidation is a purification step that is necessary for the quantification of MOAH for all vegetable oil samples. This purification step allows the elimination of olefins such as squalene, which elute within the MOAH fraction and interfere with quantification. Depending on the sample, this reaction can induce the epoxidation of a part of the MOAH or incomplete removal of the interfering olefins.

The signal area for mineral oil is calculated by subtracting riding peaks from the total area. The riding peaks can be caused by *n*-alkanes (naturally occurring hydrocarbons), terpenes, sterenes, squalene and their isomerization products as well as other substances. MOSH and MOAH are quantitated by internal standard added before analysis. Verification standards are added for monitoring proper HPLC fractionation and GC transfer conditions.

NOTE Epoxidation step can induce degradation of MOAH with three or more aromatic rings.

5 Reagents

WARNING — Reference is drawn to regulations that specify the handling of hazardous substances. Technical, organizational and personal safety measures shall be followed.

All materials shall be tested for their influence in a blank run. It is recommended to heat all glassware in an oven according to the instructions. All other materials that come into direct contact with the sample should also be heated and should not be made of polyethylene or polypropylene.

Unless otherwise stated:

— analytical pure reagents shall be used;

— water shall be either distilled or of corresponding purity;

— a solution is understood to be an aqueous solution.

5.1 Silica gel 60[1], extra pure, for column chromatography, with a particle size between 60 µm and 200 µm (70 to 230 mesh) stored in a glass bottle (protection against contamination). The silica gel is heated in an oven at 400 °C for at least 16 h and cooled in a clean desiccator (without ground grease).

5.2 Sodium sulfate, anhydrous, analytical grade, purity ≥ 99 %.

In case of contamination, heat the sodium sulfate in an oven at 400 °C for at least 16 h and allow to cool down in a clean desiccator (without ground grease).

5.3 *n*-Hexane, free of hydrocarbons in the boiling range of the *n*-alkanes C10 to C50 and other impurities such as hexane oxidation products.

Check the purity of the *n*-hexane as follows:

— mix 30 ml *n*-hexane with 25 µl of the internal standard solution (5.17) and two drops of bis(2-ethylhexyl) maleate (5.26);

— evaporate using an evaporator unit;

— dissolve the residue in 0,2 ml *n*-hexane;

— inject 50 µl into the HPLC-GC-FID system for analysis.

1) Silica gel is available from Merck, reference 7754 or 7734. It is an example of a suitable product available commercially. This information is given for the convenience of users of this document 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.

The hump signal (excluding any sharp single peaks of the solvent blank) should not exceed one-tenth of the LOQ.

NOTE Hydrocarbons in the boiling range under investigation interfere with the specific detection of mineral oil constituents in gas chromatography of the MOSH and MOAH fractions, while polar compounds such as hexane oxidation products interfere with the separation of long-chain *n*-alkanes in column chromatography on alumina.

5.4 Dichloromethane (DCM), purity ≥ 99 %.

Test the purity as for *n*-hexane (5.3) with 30 ml DCM.

5.5 Toluene.

5.6 Perylene (PER), purity > 99 %.

5.7 5-alpha-cholestane (CHO), purity ≥ 97 %.

5.8 *n*-Undecane (C11), purity ≥ 99 %.

5.9 *n*-Tridecane (C13), purity ≥ 99 %.

5.10 Tri-tert-butylbenzene (TBB), purity ≥ 97 %.

5.11 Bicyclohexyl (CYCY), purity ≥ 99 %.

5.12 1-Methyl naphthalene (1-MN), purity ≥ 95 %.

5.13 2-Methyl naphthalene (2-MN), purity ≥ 97 %.

5.14 Pentyl benzene (PB), purity ≥ 99 %.

5.15 Stock solution, mass concentrations, ρ = 5 mg/ml, 10 mg/ml or 20 mg/ml. Weigh, for example, 50 mg of C13 (5.9), 100 mg each of C11 (5.8), TBB (5.10), CYCY (5.11), 1-MN (5.12), 2-MN (5.13) and PB (5.14) as well as 200 mg CHO (5.7) and PER (5.6) to the nearest 1 mg and fill up to the mark in a 10 ml volumetric flask with toluene (5.5). Store at room temperature to keep the solutions stable. Dissolve any crystals formed during storage by gentle heating.

The verification of the start of the MOAH fraction, based on TBB, can result in losses of higher alkylated benzenes and naphthalenes, if present in some samples (i.e. cosmetics) and when the chromatographic performance of the column is limited. In such cases, di(2-ethylhexyl) benzene (DEHB) can be used in addition as verification standard and the fractionation shall be adapted.[10]

5.16 Internal standard solution 1 (ISTD1)[2], mass concentrations ρ = 150 µg/ml (C13), 300 µg/ml (C11, CYCY, PB, 1-MN, 2-MN and TBB) and 600 µg/ml (CHO and PER). Transfer 300 µl stock solution (5.15) into a 10 ml volumetric flask and fill up to the mark with toluene (5.5).

5.17 Internal standard solution 2 (ISTD2), mass concentrations ρ = 30 µg/ml (C13), 60 µg/ml (C11, TBB, CYCY, 1-MN, 2-MN and PB) and 120 µg/ml (PER and CHO) Dilute ISTD1 solution (5.16) by a factor of 5, e.g. fill up 1 000 µl ISTD1 solution (5.16) to 5 ml with *n*-hexane.

2) This standard mixture is available from, for example, Restek Corp., Cat.# 31070. It is an example of a suitable product commercially available. This information is given for the convenience of users of this document 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.

5.18 Aluminium oxide 90, alkaline, for column chromatography, 0,063 mm to 0,2 mm, activated. Heat the alumina before use for at least 16 h at 500 °C in an oven and cool down to room temperature in a cleaned desiccator (without ground grease).

5.19 *meta*-chloroperbenzoic acid (*m*CPBA), stated quantities based on a purity of about ≤ 77 %;

Commercially available *m*CPBA contains varying amounts of *m*CPBA, meta-chlorobenzoic acid and water. For purification of the reagent, remove contaminating hydrocarbons, e.g. finely suspend 5 g *m*CPBA with 200 ml *n*-hexane in a polyethylene terephthalate (PET) beaker in an ultrasonic bath and filter by using a vacuum frit. Let the purified *m*CPBA dry in a fume cupboard. Do not store in glass containers, as *m*CPBA decomposes on glass surfaces. Commercially available *m*CPBA still contains meta-chlorobenzoic acid and residual moisture to make handling in the laboratory safe. Pure *m*CPBA, on the other hand, is explosive, so isolation of *m*CPBA as a pure substance, which goes beyond the cleaning described here, is not recommended. Washing with a solvent mixture of 200 ml *n*-hexane and 20 ml DCM can remove further impurities, but also leads to significant higher losses (yields only 75 % of the initial mCPBA with a content of about 74 g to 84 g *m*CPBA per 100 g starting material in the purified product).

To determine the *m*CPBA content of the reagent, weigh about 0,2 g *m*CPBA into a PET beaker, add 50 ml distilled water and mix thoroughly. Add 5 ml concentrated acetic acid and 10 ml sodium iodide solution (10 g sodium iodide in 100 ml water). Then pre-titrate with 0,1 N sodium thiosulfate solution from dark red to light yellow. Add a few drops of starch indicator solution and titrate from dark blue to colourless at the end point (consumption usually below 20 ml).

Calculate the content w (*m*CPBA) in per cent by mass as shown by Formula (1):

$$w = \frac{N \times V \times 86{,}29 \times 100}{E} \quad (1)$$

where

N is the normality of the sodium thiosulfate solution;

V is the total volume of consumed sodium thiosulfate solution in l;

E is the mass of the reagent in g.

5.20 *m*CPBA solution in ethanol, ρ = 100 mg/ml, e.g. dissolve 1 g *m*CPBA (5.19) in 10 ml ethanol (5.28). Prepare the solution fresh every working day.

5.21 Sodium thiosulfate, anhydrous, purity > 90,0 %.

5.22 Sodium hydrogen carbonate (or sodium carbonate), anhydrous, purity > 90,0 %.

5.23 Solution for deactivation of the excess of *m*CPBA: Sodium thiosulfate and sodium carbonate solution, ρ = 50 g/l, e.g. dissolve 5 g of sodium thiosulfate and 5 g of sodium hydrogene carbonate (or sodium carbonate) in 100 ml distilled water and mix thoroughly.

5.24 Alumina column with silica gel cover. Place a filter (6.4) in a glass column (6.3), and add and compress 10 g of alumina (5.18), 3 g of silica gel (5.1) and 1 g of sodium sulfate (5.2).

5.25 Clean-up column. Place a filter (6.4) in an empty SPE glass cartridge (volume 6 ml), add 3 g of silica gel (5.1), compress and cover with 1 g of sodium sulfate (5.2).

5.26 bis (2-ethylhexyl) maleate, purity 90 %. Check the purity in a blank run.

Bis (2-ethylhexyl) maleate may be replaced by bis(2-ethylhexyl) sebacate in order to limit the risk of epoxidation process disturbance.

5.27 Standard solution of the *n*-alkanes with chain lengths of 10 to 50 in the same mass concentration for checking for discrimination against low- or high-boiling substances, ρ = 1 µg/ml. Store this solution at room temperature, otherwise C50 can crystallize.

5.28 Ethanol, absolute.

Test the purity as for *n*-hexane (5.3) with 30 ml ethanol.

5.29 Mixture of ethanol and *n*-hexane, volume fraction φ = 50 %, e.g. mix 50 ml of ethanol (5.28) with 50 ml of *n*-hexane (5.3).

5.30 Elution mixture of *n*-hexane and DCM, e.g. mix 30 ml DCM (5.4) with 70 ml *n*-hexane (5.3). Due to the volatility of DCM, the solution shall be freshly prepared.

5.31 Potassium hydroxide solution, e.g. 50 g potassium hydroxide in 100 ml distilled water, w = 33 g/100 g.

6 Apparatus

In order to achieve a sufficiently low blank level, the following process has proven to be effective: glassware (except volumetric flasks) should be heated in an oven at 430 °C for 4 h or overnight at 400 °C and kept in desiccators or other containers for use. In addition, it is recommended to:

— perform multiple determinations in different series and not directly one after another;

— not use grease for ground joints;

— not use hand cream;

— handle samples with gloves, only;

— use glassware where possible;

— check nitrogen for purity, when drying in a stream of nitrogen;

— rinse volumetric flasks, glass pipettes and other required glassware with *n*-hexane beforehand.

6.1 Analytical balance, readability 0,000 1 g, weighing accuracy 0,001 g.

6.2 Centrifuge and centrifuge tubes.

6.3 Glass column, without stopcock, 15 cm to 20 cm long and 15 mm to 20 mm inner diameter.

6.4 Filter for glass column, extracted or heated, filter made of quartz wool/glass fibre.

6.5 Glass vial, 40 ml, with polytetrafluoroethylene (PTFE) sealed screw cap.

6.6 Rotary evaporator, with vacuum and water bath at 35 °C.

Comparable devices can also be used. Take care to prevent contamination. If necessary, clean the system thoroughly between determinations.

6.7 HPLC column, e.g. LiChrospher Si 60 or Allure Silica[3], 5 µm material, 2 × 250 mm or comparable.

3) LiChrospher Si 60 and Allure Silica are examples of suitable products available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of these products.

6.8 Uncoated GC guard column, fused silica or metal capillary, e.g. HydroGuard® MXT®[4], 10 m × 0,53 mm or comparable.

NOTE The capillaries in 6.8 to 6.12 have proven to be suitable, but can be adapted to the system to meet the requirements and yield comparable results.

6.9 GC separation column, fused silica or metal capillary, programmed temperature stable up to at least 370 °C: 100 % dimethylpolysiloxane or 95 % dimethyl and 5 % phenyl methylpolysiloxane as stationary phase, length 15 m, internal diameter (ID) 0,32 mm or 0,25 mm and film thickness 0,10 µm to 0,25 µm.

6.10 Fused silica or metal capillary, deactivated, for transfer the HPLC fractions from the valve to the T-connector of the GC, 1 m long, 0,1 mm ID.

6.11 Capillary, deactivated, from the T-connector between pre- and separation column to the vapour exit.

6.12 Restriction capillary at the vapour exit, deactivated, 1 m long, ID 0,05 mm.

6.13 Syringe, 100 µl, suitable for injection of 5 to 100 µl in liquid chromatography.

6.14 Pasteur pipette made of glass.

NOTE The use of plastic pipette tips and polyethylene foil leads to increased blank levels.

6.15 Online-coupled HPLC-GC-FID system, consisting of an HPLC instrument capable of running a binary gradient, injection valve, HPLC column (6.7), ultraviolet light (UV) detector (detection wavelength: 230 nm), switching valves for column backflush and fraction transfer into GC, GC with solvent vapour exit (SVE), pneumatic control and evaluation system. In addition, an automatic control system is recommended.

6.16 Test tube shaker with temperature control and agitation (e.g. 500 r/min or comparable).

7 Sample

7.1 Sampling

Sampling is not part of this method. The sample may only be stored in glass bottles, aluminium or other materials that do not release hydrocarbons. Packaging made of paper, polyethylene or polypropylene is unsuitable. Containers made of PET or foil bags made of a high-performance polyamide such as RILSAN[5] may be used in some cases. Attention shall also be paid to the closures and sealing materials of the containers. The use of hand cream should be avoided when handling samples. The sampling shall be checked by blank runs using *n*-hexane instead of a sample.

A recommended sampling method is given in ISO 5555.

7.2 Preparation of the final sample for liquid and solid fats

Prepare the test sample in accordance with ISO 661.

Special treatments of the test sample (e.g. filtering, melting) shall be mentioned.

4) HydroGuard® MXT® is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

5) RILSAN is an example of a suitable product available commercially. This information is given for the convenience of users of this document and does not constitute an endorsement by ISO of this product.

8 Procedures

8.1 General

Depending on the type of fats and oils, the samples shall be prepared differently. The specific cases A to C are as follows:

— A: Prepare oils and fats with unknown or high content of biogenic, long-chain alkanes and unsaturated compounds[7] such as olive oil, rapeseed oil, sunflower oil and comparable samples according to 8.2 (ethanol-hexane distribution). Using two separate 10 ml fractions of the extract, run 8.3 (saponification) on the first fraction for the determination of the MOSH content according to 8.4 (Alox column), and run 8.3 (saponification) on the second fraction for the determination of the MOAH content according to 8.5 (clean-up) and 8.6 (epoxidation).

— B: Prepare oils and fats with low biogenic, long-chain alkanes and disturbing unsaturated compounds such as coconut fat, linseed oil, palm oil and comparable samples without aluminium oxide column according to 8.2 (ethanol-hexane distribution), 8.3 (saponification), 8.5 (clean-up) and 8.6 (epoxidation). Determine the MOSH and MOAH fractions from the solution obtained.

— C: For automated application of the alumina column, prepare oils and fats according to 8.2 (ethanol-hexane distribution), 8.3 (saponification), 8.5 (clean-up) and 8.6 (epoxidation). Inject into the HPLC-GC-FID system. After separation of the MOSH, which are passed to an integrated online Alox column, the MOAH fraction can be determined in the same run using a two-channel system.

Every laboratory using automated procedures shall carry out tests to ensure that the results obtained with the automated procedures do not deviate from results obtained with the manual procedure.

Unknown samples or mix oils samples may be analysed first without Alox cleanup. If the presence of interfering long-chain *n*-alkanes significantly impact the hump and do not allow a proper integration, the extract shall be re-injected using the alumina column to reduce interferences (e.g. for online Alox clean up, the same sample extract used to determine MOAH is used for the subsequent separation of long-chain *n*-alkanes with the aluminium oxide column for the determination of MOSH).

NOTE Only the manual purification method for MOSH fraction was validated during the collaborative study.

8.2 Hexane/ethanol distribution for removal of interfering substances

Weigh 3 g of the sample for oils and fats into a 40 ml centrifuge tube with screw cap. Add 30 ml of the mixture of *n*-hexane (5.3) and ethanol (5.29) and 20 µl ISTD1 (5.16) or 100 µl ISTD2 (5.17), and homogenize. Use 10 ml of this solution for the further procedure (see 8.3).

NOTE In case of oils and fats, no phase separation will be observed. Nevertheless, this step ensures a complete saponification of oils and fats. If necessary, other quantities of internal standard can be added.

8.3 Saponification

Transfer an aliquot of 10 ml (see 8.2) into another sample tube and add 3 ml potassium hydroxide solution (5.31). Saponify the solution for 30 min at 60 °C in a water bath while shaking until the solution becomes clear. Cool down, add 5 ml *n*-hexane (5.3) and 5 ml mixture of ethanol and water (a volume fraction of 1:1), shake the mixture again, transfer the lower phase after phase separation into a new vial and extract again with an additional 5 ml of *n*-hexane. Combine both *n*-hexane extracts.

Depending on the sample preparation method, continue using the solution to separate biogenic, long-chain *n*-alkanes with aluminium oxide (according to 8.4) or for epoxidation (according to 8.5 and 8.6).

NOTE The addition of ethanol after saponification enables a better phase separation and avoids foaming.