INTERNATIONAL STANDARD

ISO 15788-1

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Animal and vegetable fats and oils — Determination of stigmastadienes in vegetable oils —

Part 1:
Method using capillary-column gas chromatography (Reference method)

Corps gras d'origines animale et végétale — Dosage des stigmastadiènes dans les huiles végétales —

Partie 1: Méthode par chromatographie en phase gazeuse sur colonne capillaire (Méthode de référence)

Reference number
ISO 15788-1:1999(E)

Foreword

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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.

International Standard ISO 15788-1 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

ISO 15788 consists of the following parts, under the general title *Animal and vegetable fats and oils — Determination of stigmastadienes in vegetable oils*:

— *Part 1: Method using capillary-column gas chromatography*

— *Part 2: Method using high-performance liquid chromatography*

Annex A of this part of ISO 15788 is for information only.

International Organization for Standardization
Case postale 56 • CH-1211 Genève 20 • Switzerland
Internet iso@iso.ch

Printed in Switzerland

Introduction

Significant amounts of hydrocarbons are formed in vegetable oils as a consequence of thermal treatments during the refining processes [1]. Among these hydrocarbons, 3,5-stigmastadiene, a steroidal compound, is the most abundant in all refined vegetable oils since it is derived from β-sitosterol by dehydration [2]. The 3,5-stigmastadiene is produced together with minor amounts of the 2,4-isomer and both substances give a single well-defined gas-chromatographic peak when the hydrocarbon fraction is analysed on a low polar column [3]. Therefore, the sum of both isomers can be easily quantified by gas-chomatographic analysis of the steroidal hydrocarbon fraction [2], [4].

For instance, for virgin olive oils, the usual oil production processes (pressure or centrifuging) do not produce measurable amounts of stigmastadienes (less than 0,01 mg/kg). In crude olive residue oil, small concentrations of stigmastadienes are found (ranging between 0,2 mg/kg and 3 mg/kg) due to the high temperatures applied during the drying of the raw olive residue.

In the refining processes, stigmastadienes are formed in all the steps involving high temperatures, such as bleaching and deodorizing, but more amounts are formed in the first step using acid bleaching earth than in the second [2]. Depending on the conditions applied during the refining process, commercial refined vegetable oils show stigmastadiene concentrations ranging between 1 mg/kg and 120 mg/kg and, therefore, the assessment of stigmastadienes allows not only the identification of thermally treated oils but also the detection of minor amounts of refined vegetable oils in virgin oils.

A method for the determination of stigmastadienes and the results of a collaborative study carried out under the auspices of the International Olive Oil Council were presented to the International Union of Pure and Applied Chemistry (IUPAC) Commission on Oils, Fats and Derivatives. The stigmastadiene content has been adopted as an identity criterion for virgin olive oils and the resulting analytical method has been adopted as an international standard method.

Animal and vegetable fats and oils — Determination of stigmastadienes in vegetable oils —

Part 1:
Method using capillary-column gas chromatography (Reference method)

1 Scope

This part of ISO 15788 specifies a method for the determination of stigmastadienes in virgin vegetable oils containing low concentrations of these hydrocarbons, particularly in virgin olive oil.

This method is applicable to all vegetable oils although measurements are reliable only where the content of these hydrocarbons lies between 0,01 mg/kg and 4,0 mg/kg. The method is suited to detecting the presence of refined vegetable oils (olive, olive pomace, sunflower, palm, etc.) in virgin olive oils, since refined oils contain stigmastadienes and cold-extracted oils do not.

2 Principle

Unsaponifiable matter is isolated. The steroidal hydrocarbon fraction is separated by column chromatography on silica gel and analysed by capillary gas chromatography.

3 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

3.1 Hexane, or mixture of alkanes of boiling point from 65 °C to 70 °C, distilled with a rectifying column.

The hexane shall be redistilled to remove impurities.

3.2 Ethanol, 96 % (volume fraction).

3.3 Anhydrous sodium sulfate.

3.4 Alcoholic potassium hydroxide solution, of concentration 50 g per 500 ml.

Add 10 ml of water to 50 g potassium hydroxide, stir, and then dilute the mixture with ethanol to 500 ml.

Alcoholic potassium hydroxide solution turns brown on standing. It should be prepared freshly each day and kept in well-stoppered dark glass bottles.

3.5 Silica gel 60[1], for column chromatography, 70 to 230 mesh.

Usually, silica gel can be used directly from the container without any treatment. However, some batches of silica may show low activity resulting in poor chromatographic separations. Under these circumstances, the silica gel should be treated in the following way.

Activate the silica gel by heating for at least 4 h at 550 °C. After heating, place the silica gel in a desiccator while the gel is cooling and then transfer the silica gel to a stoppered flask. Add 2 % of water and shake until no lumps can be seen and the powder flows freely.

If batches of silica gel result in chromatograms with interfering peaks, the silica gel should be treated as above. An alternative would be the use of extra pure silica gel 60[1].

3.6 3,5-Cholestadiene [2], 99 % purity (mass fraction), stock solution in hexane, of concentration 10 mg per 50 ml.

3.7 3,5-Cholestadiene, standard solution in hexane, of concentration of 1 mg per 50 ml, obtained by dilution of the stock solution (3.6).

NOTE If kept at a temperature of below 4 °C, solutions (3.6) and (3.7) will not deteriorate over a period of at least 4 months.

3.8 *n*-Nonacosane, solution in hexane, of concentration approximately 10 mg per 100 ml.

3.9 3,5-Stigmastadiene[3] (24-ethylcholesta-3,5-diene), solution in hexane, of concentration approximately 10 mg per 100 ml.

3.10 Carrier gas, for chromatography: helium or hydrogen of 99,9990 % purity (mass fraction).

3.11 Auxiliary gases, for flame ionization detector: hydrogen of 99,9990 % purity (mass fraction), and purified air.

4 Apparatus

Usual laboratory apparatus and, in particular, the following.

4.1 Flasks, of 250 ml capacity, suitable for use with a reflux condenser.

4.2 Separating funnel, of 500 ml capacity.

4.3 Round-bottomed flasks, of 100 ml capacity.

4.4 Rotary evaporator.

4.5 Glass chromatography column of 1,5 cm i.d. and 50 cm length, with Teflon tap and a plug of glass wool fibre or sintered glass disc at the bottom.

To prepare a silica gel column, pour hexane into the chromatography column to a depth of approximately 5 cm and then fill with a slurry of silica gel in hexane (15 g in 40 ml) with the help of hexane portions. Allow to settle and finish the settling by applying slight vibration. Add anhydrous sodium sulfate (3.3) to a height of approximately 0,5 cm. Finally elute the excess hexane.

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[1] Product supplied by Merck, ref. 7734 or similar and ref. 7754 (for extra pure silica gel 60).

[2] Product supplied by Sigma.

[3] Product supplied by Chiron A.S., Heimdal, Norway.

[1],[2] and [3] This information is given for the convenience of users of this International Standard and does not constitute an endorsement by ISO of these products. Equivalent products may be used if they can be shown to lead the same results.

4.6 Gas chromatograph, with a flame ionization detector, split or on-column injector, and oven programmable to within ± 1 °C.

4.7 Fused silica capillary columns, for gas chromatography (0,25 mm or 0,32 mm i.d. by 25 m length) coated with 5 % phenylmethylsilicone phase, 0,25 µm film thickness.

NOTE Other columns of similar or lower polarity can be used.

4.8 Integrator-recorder, with possibility of valley-valley integration mode.

4.9 Microsyringe, of 5 µl to10 µl capacity, for gas chromatography, with cemented needle.

4.10 Electrical heating mantle, or **hot plate**.

5 Procedure

5.1 Preparation of unsaponifiable matter

5.1.1 Weigh 20 g ± 0,1 g of oil into a 250 ml flask (4.1). Add 1 ml of the internal standard solution of 3,5-cholestadiene (containing 20 µg) and 75 ml of alcoholic potassium hydroxide (3.4). Fit a reflux condenser and heat the solution to slight boiling for 30 min. Remove the flask containing the sample from the heat and allow the solution to cool slightly (do not allow it to cool completely or the sample will set). Add 100 ml of water and transfer the solution to a separating funnel (4.2) with the aid of 100 ml of hexane. Shake the mixture vigorously for 30 s and allow it to separate.

If an emulsion is produced which does not rapidly separate, add small quantities of ethanol.

5.1.2 Transfer the lower aqueous phase to a second separating funnel and extract again with 100 ml of hexane. Once more run off the lower phase and wash the hexane extracts (combined in another separating funnel) three times with 100 ml each time of a mixture of ethanol/water [50 ml/100 ml (volume fraction)] until neutral pH is reached.

5.1.3 Pass the hexane solution through anhydrous sodium sulfate (50 g), wash with 20 ml of hexane and evaporate in a rotary evaporator at 30 °C under reduced pressure until dryness.

5.2 Separation of steroidal hydrocarbon fraction

5.2.1 Transfer the residue to the fractioning column with the aid of two 1 ml portions of hexane. Run the sample onto the column by allowing the solution level to drop to the top of the sodium sulfate and start the chromatographic elution with hexane at a flowrate of 1 ml/min approximately. Discard the first 25 ml to 30 ml of the eluate and then collect the following 40 ml fraction. After collection, transfer this fraction to a 100 ml round-bottomed flask (4.3).

The first fraction contains saturated hydrocarbons [Figure 1a)] and the second fraction the steroidal ones. Further elution provides squalene and related compounds. To achieve a good separation betwen saturated and steroidal hydrocarbons, the optimization of fraction volumes is required. For this, the volume of the first fraction should be adjusted so that when the second fraction is analysed the peaks representing the saturated hydrocarbons are low [Figure 1c)]; if they do not appear but the intensity of the standard peak is low, the volume should be reduced. However, a complete separation between the components of the first and second fractions in unnecessary, as there is no overlapping of peaks during GC analysis if the GC conditions are adjusted as indicated in 5.3.1.

NOTE The optimization of the volume of the second fraction is generally not needed as a good separation exists with the other components. Nevertheless, the presence of a large peak at a retention time approximately 1,5 min lower than that of the standard is due to squalene, and it is indicative of a poor separation.

5.2.2 Evaporate the second fraction in a rotary evaporator at 30 °C under reduced pressure until dryness, and immediately dissolve the residue in 0,2 ml of hexane. Keep the solution in the refrigerator until analysis.

Residues from steps 5.1.3 and 5.2.2 shall not be kept dry or at room temperature. As soon as they are obtained, add the solvent and keep the solutions in the refrigerator.

5.3 Gas chromatography

5.3.1 Working conditions for split injection

These shall be as follows:

— injector temperature, 300 °C;

— detector temperature, 320 °C;

— integrator-recorder: the parameters for integration should be fixed so as to give a correct assessment of the areas; valley-valley integration mode is recommended;

— sensitivity: about 16 times the minimum attenuation;

— amount of solution injected, 1 µl;

— oven programming temperatures: initial, 235 °C for 6 min, and then rising at 2 °C/min up to 285 °C;

— injector with 1: 5 flow divider;

— carrier gas: helium or hydrogen at a pressure of about 120 kPa and 85 kPa, respectively.

These conditions may be adjusted in accordance with the characteristics of the gas chromatograph apparatus and the column to give chromatograms meeting the following requirements:

— internal standard peak within approximately 5 min of the time given in 5.3.2;

— the internal standard peak should be at least 80 % of the full scale.

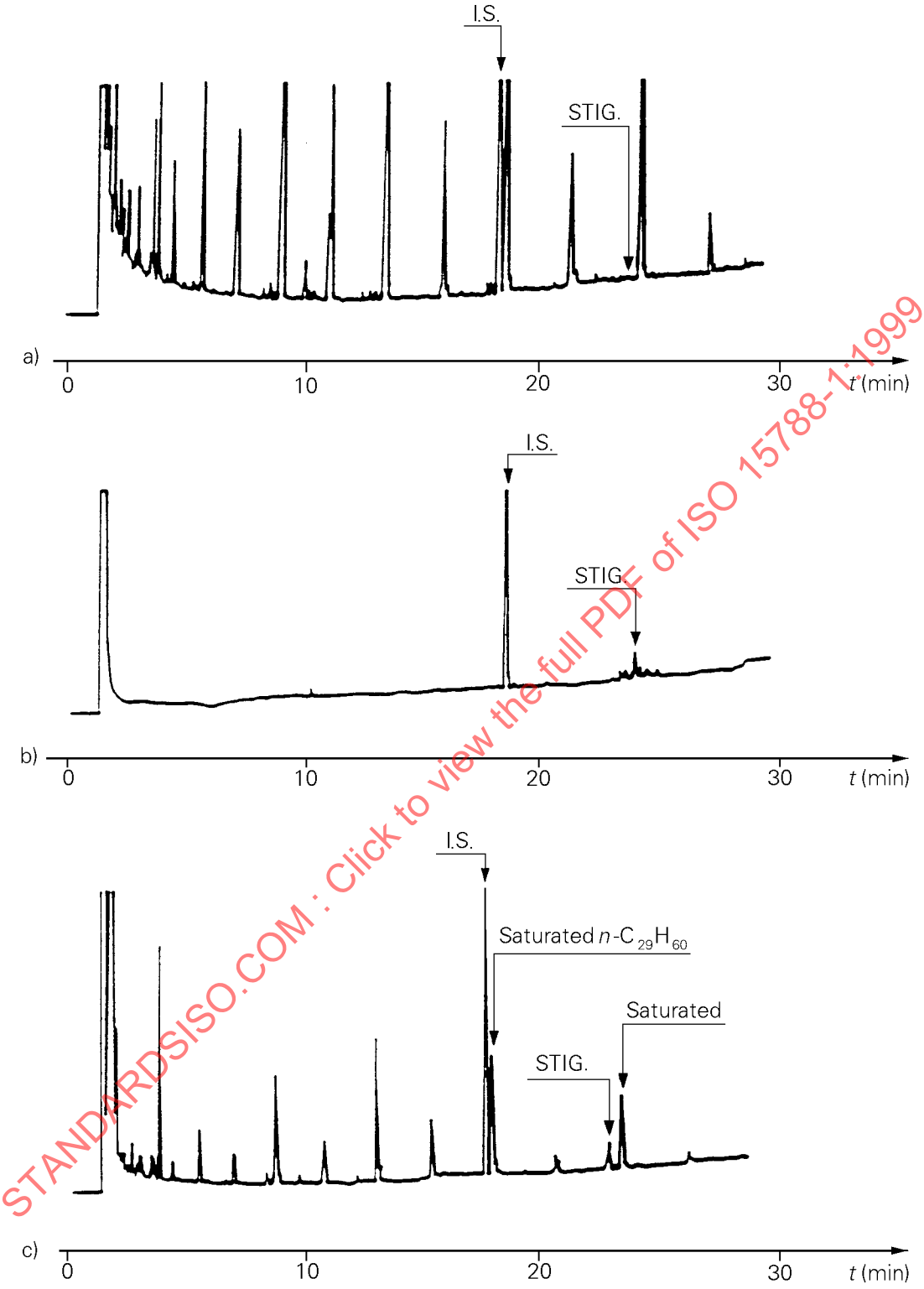
The gas chromatographic system shall be checked by injecting a mixture of the stock solution of 3,5-cholestadiene (3.6) and *n*-nonacosane solution (3.8). The 3,5-cholestadiene peak shall appear before the *n*-nonacosane [Figure 1c)]. If that does not occur, two actions can be undertaken: reduce the oven temperature and/or use a less-polar column.

5.3.2 Peak identification

If helium is used as a carrier gas, the internal standard peak appears at approximately 19 min and the stigmastadienes peak at a relative retention time of 1,29 [Figure 1b)]. If hydrogen is used, the internal standard peak appears at approximately 13 min and the stigmastadienes peak at relative retention time of 1,35. Reference for the stigmastadienes peak can be obtained by analysing a solution of 3,5-stigmastadiene (3.9).

NOTE If 3,5-stigmastadiene (24-ethyl-cholesta-3,5-diene) is not available, a chromatographic reference for stigmastadienes can be obtained by analysing 1 g to 2 g of a refined vegetable oil. Stigmastadienes give a significant and easily identifiable peak.

The stigmastadienes peak is a single chromatographic peak coming from a mixture of 3,5-stigmastadiene and minor amounts of the 2,4-isomer. Nevertheless, if the column is too polar or shows a high resolving power, the 2,4-isomer can appear as a small peak before and close to that of 3,5-stigmastadiene (Figure 2). In order to ensure that the both stigmastadienes are eluted as one peak, it is advisable to replace the column by one which is less polar or has a wider internal diameter.



a) First fraction (30 ml) from a virgin oil, spiked with standard (I.S.).

b) Second fraction (40 ml) from an olive oil containing 0,10 mg/kg of stigmastadienes.

c) Second fraction (40 ml) containing a small proportion of the first fraction.

Figure 1 — Gas chromatograms obtained from olive oil samples analysed on a fused silica capillary column (0,25 mm i.d. by 25 m) coated with 5 % phenylmethylsilicone, 0,25 µm film thickness

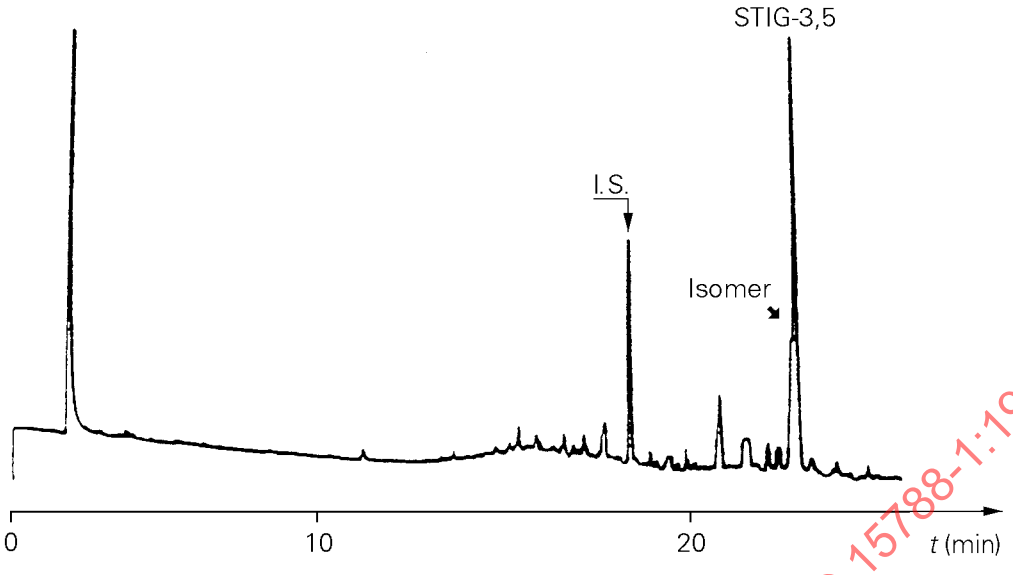


Figure 2 — Gas chromatogram obtained from a refined olive oil sample analysed on DB-5 column showing the isomer of 3,5-stigmastadiene

6 Calculation

The stigmastadiene content, w, in milligrams per kilogram, is determined according to the equation:

$$w = \frac{A_s \times m_c}{A_c \times m_o}$$

where

A_s area of stigmastadienes peak (if the peak is split up, take the sum of the areas of the 3,5- and 2,4-isomers);

A_c is the area of the internal standard (3,5-cholestadiene);

m_c is the mass of standard (3,5-cholestadiene) added, in micrograms;

m_o is the mass of oil taken, in grams.

The response factor of the internal standard 3,5-cholestadiene in relation to that of the stigmastadienes is equal to 1.

Report the result to two decimal places.

7 Precision

7.1 Interlaboratory test

Details of an interlaboratory test on the precision of the method are summarized in annex A. The values derived from this interlaboratory test may not be applicable to concentration ranges and matrices other than those given.

7.2 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 short intervals of time, will in not more than 5 % of cases be greater than the repeatability limit r, calculated from the following equation:

$$r = 0{,}20\,\overline{w}$$

where $\overline{w}$ is the mean stigmastadienes content in the range of 0,10 mg/kg to 1,0 mg/kg.

7.3 Reproducibility

The absolute difference between two single test results, obtained with the same method on identical test material in different laboratories with different operators using different equipment, be greater than the reproducibility limit R calculated from the following equation:

$$R = 0{,}41\,\overline{w}$$

where $\overline{w}$ is the mean stigmastadienes content in the range of 0,10 mg/kg to 1,0 mg/kg.

7.4 Sensitivity

The sensitivity of the method is demonstrated by the low values for r and R at the low concentrations studied. The limit of detection is 0,01 mg/kg.

8 Test report

The test report shall specify:

— all information necessary for the complete identification of the sample;

— the sampling test method used, if known;

— the test method used, with reference to this International Standard;

— all operating details not specified in this International Standard, or regarded as optional, together with details of any incidents which may have influenced the test results;

— the test results obtained; or

— if the repeatability has been checked, the final quoted result obtained.