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

Part 2: Method using high-performance liquid chromatography (HPLC)

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

Partie 2: Méthode par chromatographie liquide à haute performance (CLHP)

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Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. Draft International Standards adopted by the technical committees are circulated to the member bodies for voting. Publication as an International Standard requires approval by at least 75 % of the member bodies casting a vote.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 15788-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

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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 (Reference method)*

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

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

Part 2: Method using high-performance liquid chromatography (HPLC)

1 Scope

This part of ISO 15788 specifies a method for the determination of steradienes, especially stigmastadienes. Steradienes are formed by dehydration of sterols during bleaching and also partially during steam washing and deodorization. The method is also suitable as a screening method to detect the presence of refined vegetable oils in virgin oils such as virgin olive oil.

NOTE ISO 15788-1 is the reference method for the determination of stigmastadienes in vegetable oils whilst this part of ISO 15788 can be used as a rapid screening method. In view of the precision of this method (see Annex A), samples of virgin olive oils close to the limit adopted by international regulations (IOOC, EC) can be verified by the GLC method given in ISO 15788-1.

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2 Normative references

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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 3696:1987, *Water for analytical use — Specification and test method*

ISO 12228, *Animal and vegetable fats and oils — Determination of individual and total sterols content — Gas chromatographic method*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

3.1
stigmastadienes content
that part of the stigmastadienes separated by liquid chromatography under the conditions specified in this International Standard

NOTE It is expressed in milligrams per kilogram.

3.2
steradienes content
that part of all the steradienes separated by liquid chromatography under the conditions specified in this International Standard

NOTE It is expressed in milligrams per kilogram.

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4 Principle

Steradienes are separated from the major part of other lipids as non-polar fat compounds, using petroleum ether on a silica gel column. The petroleum ether eluate is concentrated then analysed by RP-18-HPLC and UV-detection at 235 nm. They are quantified, depending on the kind of sample, using an internal or external standard.

5 Reagents

WARNING — Attention is drawn to the regulations which prescribe the handling of dangerous matter. Technical, organizational and personal safety measurements shall be followed.

Use only reagents of recognized analytical grade, unless otherwise stated.

5.1 Water, of at least grade 1 according to ISO 3696:1987.

5.2 Silica gel 60 for column chromatography, particle size 0,063 mm to 0,200 mm, or 0,063 mm to 0,100 mm[1)], with a water content of 2 g per 100 g.

Dry the silica gel in a porcelain dish for 12 h at 160 °C in a drying oven and cool to room temperature in a desiccator. For adjusting the silica gel to a water content of 2 g per 100 g, weigh (to the nearest 1 g) 98 g of the dried silica gel in a conical flask with ground glass stopper and add 2 g of water (weighed to the nearest 0,01 g). Shake vigorously for 1 min and allow the silica gel to stand overnight in an airtight vessel.

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5.3 Petroleum ether, boiling range 40 °C to 60 °C.

5.4 Acetonitrile, chromatography grade.

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5.5 *tert*-Butyl methyl ether, chromatography grade.

5.6 Isooctane.

5.7 Δ3,5-Cholestadiene[2)], with a known purity of at least 95 g per 100 g.

Check the purity of the cholestadiene standard by gas chromatography using 5α-cholestane as internal standard. For this test, see the conditions for the gas chromatographic method for the determination of sterols specified in ISO 12228. The response factor of the flame ionization detector should be 1,0. Take into consideration the obtained concentration during the determination of the steradienes.

5.8 Stock solutions and standard solutions

5.8.1 Δ3,5-Cholestadiene stock solution, of concentration 1 mg/ml.

Weigh, to the nearest 0,1 mg, 50,0 mg of Δ3,5-cholestadiene into a 50 ml measuring flask. Dissolve and dilute to the mark with *tert*-butyl methyl ether (5.5).

5.8.2 Δ3,5-Cholestadiene standard solution for HPLC

1) For example, No. 7734 or 15101 from E. Merck, 64271 Darmstadt, Germany.

2) For example, No. C6012 from Sigma Chemie GmbH, Grünwalder Weg 30, 82041 Deisenhofen, Germany.

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5.8.2.1 External standard solution, of concentration 10 µg/ml.

Pipette 100 µl of the Δ3,5-cholestadiene stock solution (5.8.1) into a 10 ml measuring flask and fill up to the mark with acetonitrile/*tert*-butyl methyl ether (5.8.4).

A 20 µl aliquot of this solution contains 0,20 µg. This is injected into the HPLC column. The concentration of the standard solution depends on the nature of the oil to be analysed. In the case of virgin oils with stigmastadienes contents of less than 0,5 mg/kg, the concentration of the external standard solution shall be 0,2 µg/ml.

5.8.2.2 Internal standard solution, of concentration 2 µg/ml.

Pipette 100 µl of the Δ3,5-cholestadiene stock solution (5.8.1) into a 50 ml measuring flask and dilute to the mark with petroleum ether (5.3).

The required concentration of the standard solution depends on the nature of the oil to be analysed. In the case of virgin oils with stigmastadienes contents of less than 0,5 mg/kg, the concentration of the internal standard solution shall be 0,2 µg/ml.

5.8.3 5α-Cholestane standard solution for GC, of concentration 1 mg/ml.

Weigh, to the nearest 0,1 mg, 50,0 mg of 5α-cholestane[3)] into a 50 ml measuring flask and dilute to the mark with isooctane (5.6). Use this solution for the determination of the purity of the Δ3,5-cholestadiene standard (5.8.2) as follows.

Pipette 1 ml each of the 5α-cholestane standard solution and the Δ3,5-cholestadiene stock solution (5.8.1) into a 10 ml measuring flask (split injection) or into a 50 ml measuring flask (on-column injection) and dilute to the mark with isooctane.

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5.8.4 Acetonitrile/*tert*-butyl methyl ether, [50:50 (by volume)].

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5.8.5 Mobile phase for HPLC: acetonitrile/*tert*-butyl methyl ether, [70:30 (by volume)], degassed.

5.9 Cholestane, with a known purity of at least 95 g per 100 g.

6 Apparatus

Usual laboratory apparatus and, in particular, the following.

6.1 Cotton wool or **glass wool.**

The cotton wool may be defatted by extraction for 8 h with petroleum ether.

6.2 Chromatographic column, made of glass, of 10 mm internal diameter and 150 mm length, with a 25 ml reservoir.

6.3 Taper-shaped flasks, of capacity 25 ml.

6.4 Volumetric flasks, of capacities 5 ml, 10 ml and 50 ml.

6.5 Beakers, in several sizes.

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3) For example, No. C8003 from Sigma Chemie GmbH, Grünwalder Weg 30, 82041 Deisenhofen, Germany.

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6.6 HPLC system, consisting of a pump, a sample injecting device (20 µl and 100 µl loop), a UV detector for measurements at 235 nm, and an integration system.

6.7 HPLC column, of length 250 mm, internal diameter 4,0 mm or 4,6 mm, with reverse-phase type RP 18 filling, and particle size 5 µm[4]).

6.8 Autosampler vials, of suitable capacity.

6.9 Rotary evaporator, with water bath.

7 Sampling

It is important the laboratory receive a sample which is truly representative and has not 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.

8 Preparation of test sample

8.1 General

Remove water from the fat sample before the analysis, if necessary, by heating approximately 5 g of the sample to 100 °C for a short time, then centrifuging.

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Plug the end of the chromatographic column with a small piece of wool (6.1).

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Add 5 g of silica gel (5.2) without solvent to the column and pack by tapping the column softly on a wooden board.

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8.2 External standard method

Weigh, to the nearest 1 mg, about 500 mg of the sample in a small beaker. Dissolve it in 2 ml of petroleum ether and pour the solution onto the column with the stopcock open. Rinse the beaker twice with 2 ml portions of petroleum ether each time.

As soon as the solvent has drained to the top of column packing, elute the non-polar substances with 20 ml of petroleum ether and collect them in the flask (6.3).

Evaporate the solvent to dryness on a rotary evaporator and dissolve the residue in 500 µl of acetonitrile/*tert*-butyl methyl ether (5.8.4).

8.3 Internal standard method

When using this method, ensure that the sample does not contain small quantities of Δ3,5-cholestadiene (e.g. refined oils).

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4) For example, Supersphere 100, RP 18 end-capped, No. 1.16858 from E. Merck, 64271 Darmstadt, Germany.

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Weigh, to the nearest 1 mg, about 500 mg of the sample in a small beaker. Add 1,0 ml of the Δ3,5-cholestadiene internal standard solution. Dissolve it in 2 ml of petroleum ether and pour the solution onto the column with the stopcock open. Rinse the beaker twice with 2 ml of petroleum ether each time.

As soon as the solvent has drained to the top of column packing, elute the non-polar substances with 20 ml of petroleum ether and collect them in the taper-shaped flask (6.3).

Evaporate the solvent to dryness on a rotary evaporator and dissolve the residue in about 500 µl of acetonitrile/*tert*-butyl methyl ether (5.8.4).

9 Procedure

9.1 High-pressure liquid chromatography (HPLC)

The following conditions have been found to be suitable:

stationary phase: RP-18, 5 µm;

column dimensions: 250 mm × 4,6 mm;

mobile phase: acetonitrile/*tert*-butyl methyl ether (5.8.5);

flow rate: 1 ml/min;

injection volume: 20 µl to 100 µl (depending on the expected concentration);

detection: UV, 235 nm.

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9.2 Identification of steradienes

Carry out the identification and assignment of peaks by means of relative retention times given in Table 1 (see also the chromatograms in Annex B). Cholestadiene serves as reference substance which elutes after 20 min to 25 min.

Table 1 — Relative retention time for sterol derivatives

Sterol derivative	Relative retention time (RRT)
Cholestadiene	1,00
Stigmastatriene	1,05
Campestadiene	1,07
Stigmastadiene	1,15

NOTE Depending on the fats or oils analysed, further steradienes and steratrienes are to be expected. An identification of the peaks can be made by injection of a sample of a refined oil or fat or by a self-made stigmastadiene standard. Under the given chromatographic conditions, the chromatogram of a virgin (unbleached) oil does not show any peaks in that part of the chromatogram where the steradienes are to be expected.

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