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Determination of fatty acid methyl esters (*cis* and *trans*) and squalene in olive oil and other vegetable oils by gas chromatography

*Détermination des esters méthyliques d'acides gras (*cis *et* trans*) et du squalène dans l'huile d'olive et d'autres huiles végétales par chromatographie en phase gazeuse*

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Contents

Page

Foreword

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The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular, the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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For an explanation of the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT), see www.iso.org/iso/foreword.html.

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

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.

Determination of fatty acid methyl esters (*cis* and *trans*) and squalene in olive oil and other vegetable oils by gas chromatography

1 Scope

This document specifies the determination of the fatty acid methyl esters (FAME) and squalene in olive oil and other vegetable oils by gas chromatography (GC).

This document is applicable to the determination of FAME from C12 to C24, including saturated, *cis*- and *trans*-monounsaturated, *cis*- and *trans*-polyunsaturated FAME and squalene.

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
squalene content
mass fraction of squalene in the sample

Note 1 to entry: As determined under the conditions specified in this document.

Note 2 to entry: The squalene content is expressed in milligrams per kilogram of oil, without a decimal place.

3.2
fatty acids methyl esters
FAME
percentage area of triglyceride fatty acids as well as free fatty acids that has been methylated in the sample

Note 1 to entry: As determined under the conditions specified in this document.

Note 2 to entry: The FAME are expressed as percentage area of FAME (% area of individual fatty acid per 100 % area of total FAME present in the sample taken).

4 Principle

FAME are formed by *trans*-esterification with methanolic solution of potassium hydroxide at room temperature. FAME from C12 to C24, including saturated, *cis*- and *trans*- monounsaturated and *cis*- and

trans- polyunsaturated FAME are then determined by capillary GC. In the same method, the amount of squalene is determined in mg/kg using a standard squalene calibration curve.

For oils with FFA > 2,0 %, a prior silica gel solid phase extraction (SPE) clean-up is recommended.

5 Reagents and materials

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

During the analysis, unless otherwise stated, use only reagents of recognized analytical grade and distilled water or water of equivalent purity.

5.1 Potassium hydroxide, (≥85 g/100 g potassium hydroxide).

5.2 Methanol, containing not more than 0,5 % mass fraction water.

5.3 Methanolic potassium hydroxide solution (2 M). Dissolve, with gentle heating, 13,1 g potassium hydroxide (5.1) in 100 ml absolute methanol (5.2).

5.4 Heptane. Heptane may be replaced by iso-octane (2,2,4-trimethyl pentane, chromatography grade).

5.5 Sodium sulfate, anhydrous, ≥ 99 %.

5.6 Internal standard solution. For the quantification of fatty acids, in g/100 g, the use of an internal standard is necessary.

NOTE The use of an external calibration with reference mixtures of different fatty acids is also possible.

Prepare an internal standard solution of 5,0 mg/ml heneicosanoic acid methyl ester (C21:0 FAME) in heptane (5.4). Use C21:0 FAME with a purity of not less than 99 %.

5.7 Reference standard

5.7.1 Fatty acid methyl esters

Use a suitable mixture of FAME and squalene covering the range of analytes expected in the sample material.

For identification, use a mixture of methyl esters of pure fatty acids, in particular *cis*- and *trans*-isomers of octadecenoic (oleic), *trans*-isomers of octadecadienoic (linoleic) and octadecatrienoic (α-linolenic) acids, together with a reference chromatogram.

Use pairs of standards such as C18:3/C20:1 and C23:0/squalene to ensure critical pair separation and identification.

5.7.2 Squalene

5.7.2.1 For the quantification of squalene, a standard curve is necessary.

5.7.2.2 Standard stock solution: 10,0 mg/ml of squalene (>99 % purity) in heptane (5.4).

5.7.2.3 Standard solutions: Using 5.7.2.2 prepare a series of solutions containing 0,5 to 5,0 mg/ml of squalene in heptane (5.4). Inject 1 µl of each standard solution (5.7.2.3) into the chromatograph and

record the resulting chromatograms. Construct a standard curve by plotting the content of squalene (in µg/ml) against the peak area.

To check for loss of squalene during FAME preparation, a comparison of the results before and after methylation is recommended.

5.8 Carrier gas. Inert gas (helium or hydrogen), thoroughly dried and with an oxygen content <10 mg/kg.

5.9 Auxiliary gases:

a) **Hydrogen** (purity ≥ 99,9 %), free from organic impurities.

b) **Air or oxygen**, free from organic impurities.

c) **Nitrogen** (purity > 99 %).

6 Apparatus

The usual laboratory equipment and, in particular, the following shall be used.

6.1 Analytical balance, capable of weighing to the nearest 0,001 g

6.2 Graduated or automatic pipette, capacity 0,1 to 2 ml

6.3 Pipette tip, capacity 0,1 to 2 ml

6.4 Screw-top test tubes, capacity 10 ml with cap fitted with a PTFE liner.

6.5 Vortex.

6.6 GC vials and caps, capacity 2 ml.

6.7 Gas chromatograph for capillary column analysis, split injector, flame ionization detector (FID) and suitable integration system.

6.8 Capillary column, for GC. Length 100 m and 0,25 mm internal diameter coated with SP-2560[1)] or CP Sil 88[1)], 100 % cyanopropyl-silicone stationary phase with a film thickness of 0,25 µm. Other columns of similar polarity and selectivity may also be used.

Condition the column by temperature programming the oven at 3 °C/min from ambient temperature to a temperature 10 °C below the decomposition limit of the stationary phase. Maintain the oven at this temperature for 1 h until stabilization of the baseline. Return it to 165 °C for use under the conditions of the method.

7 Sample

7.1 Sampling

Sampling is not part of the method specified in this document. A recommended sampling method is given in ISO 5555. It is important that the laboratory receives a sample which is representative and has not been damaged or changed during transport or storage.

1) SP-2560 and CP Sil 88 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.

7.2 Preparation of test sample

Prepare the test sample in accordance with ISO 661.

8 Procedure

8.1 Preparation of FAME samples

8.1.1 Weigh 0,1 g of sample into a 10 ml test tube (6.4). When testing for squalene or mass of fatty acid methyl ester, record the mass.

For samples with free fatty acid levels > 2,0 %, prior clean-up to remove free fatty acids is recommended. A suitable method is as follows:

— Rinse a 1 g silica gel cartridge (6 ml) in a vacuum elution apparatus with 6 ml of hexane without vacuum.

— Load a sample of oil (approximately 0,12 g in 0,5 ml hexane) onto the column.

— Using the vacuum, load the column and then elute with a total of 10 ml of hexane/diethyl ether (87:13 v/v).

— Combine the fractions and evaporate to dryness under reduced pressure at room temperature.

— Dissolve the residue in hexane and transfer into a tube and take to dryness under nitrogen.

— Use in 8.1.1.

8.1.2 Add 200 µl internal standard solution (5.6) into the same test tube (6.4) when testing for mass of FAME.

The internal standard solution (5.6) is not necessary for squalene determination, but a squalene standard curve is required.

8.1.3 Add 2 ml of heptane (5.4) to all samples and lightly shake to mix.

8.1.4 Add 0,2 ml of methanolic potassium hydroxide solution (5.3) to all samples.

8.1.5 Cap and vortex (6.5) for 30 s.

8.1.6 Leave to stratify until the upper solution becomes clear (approximately 30 min).

8.1.7 Transfer the upper layer containing the methyl esters and squalene into the GC vial (6.6).

Na_2SO_4 may be added to dry the upper layer, if necessary. Sample transfer is not required if autosampler needle is adjusted to stop above the Na_2SO_4 layer

8.1.8 Analyse the samples by GC as soon as possible or keep the solution in the refrigerator for no more than 12 h before analysis.

8.2 Gas chromatography

8.2.1 General

The following conditions have been proven to be suitable for the separation of FAME (C12 to C24) and squalene:

— Injector temperature: 250 °C.

— Detector temperature: 250 °C.

— Oven temperature: The initial temperature is set at 165 °C for 30 min, then programmed to increase at a rate of 2 °C/min to 200 °C and the final temperature is maintained for a further 12 min.

— Carrier gas hydrogen: column head pressure, 26 psi; 1,0 ml/min; constant flow.

— Split ratio: 1:100.

— Injection volume: 1 µl.

An example chromatogram is shown in Annex A.

8.2.2 System suitability

Using an injection size of 1 µl, check the performance of the column (6.8) using the reference standard solution (5.7.1 and 5.7.2). Adjust the test portion size, test portion concentration or oven temperature (in 1° increments) if necessary, to produce a chromatogram with optimal separation that matches the chromatogram provided with the mixture of FAME and squalene used in the reference standard solution. Pay particular attention to the separation and identification of critical pairs such as C18:3/C20:1 and C23:0/squalene. Isomers of both C16:1c and C18:1c may be partially separated depending on the column used and chromatographic conditions. However, for this analysis they should be integrated as a single peak for C16:1c and a single peak for C18:1c.

9 Expression of results

9.1 Quantitative analysis

9.1.1 FAME, by area %

For the scope of this document, for samples in which significant amounts of components below C12:0 are absent, calculate the content of each of the individual fatty acids from C12:0 to C24:0, present in the chromatogram, expressed as a percentage by area of methyl esters, as shown by Formula (1):

$$W_i = (A_i / \sum A) \times 100 \quad (1)$$

where

W_i is the content individual fatty acids methyl ester;

A_i is the area under the peak of the individual FAME *i*;

$\sum A$ is the sum of the areas under all the peaks of all the individual FAME.

The results are expressed to two decimal places.

9.1.2 FAME, by mass (g/100 g)

If the quantification by mass of an individual fatty acid methyl ester (i) is needed, the calculation shown by Formula (2) should be applied. For example, the calculation of linolenic acid content (in g/100 g) using an internal standard:

$$L = [(A_i \times m_{is}) / (A_{is} \times M)] \times 0{,}1 \quad (2)$$

where

L is the linolenic acid content, in g/100 g

A_i is the area under the peak of linolenic acid in the sample;

m_{is} is the mass of internal standard added, in mg;

A_{is} is the area under the peak of internal standard;

M is the mass of the sample taken for the determination, in g;

0,1 is used to convert the result to g/100 g.

Refer to Annex A for an example of a chromatogram.

Refer to Annex B for quantitative analysis using correction factors, in the presence of fatty acids methyl esters with less than 12 carbon atoms.

9.1.3 Squalene

9.1.3.1 Calculation using a standard calibration curve

Calculate the content of squalene present in the sample, expressed as mg/kg oil, as shown by Formula (3).

$$C_s = (C_c \times V)/m \quad (3)$$

where

C_s is the concentration of squalene in mg/kg oil;

C_c is the content of squalene from the standard calibration curve, in µg/ml;

V is the final volume of the sample preparation, in ml;

m is the mass of the sample taken used in the sample preparation, in g (8.1.1).

Refer to Annex A for an example chromatogram.

To determine the ratio of fatty acid and squalene content both should be expressed on the same basis (mg/kg) or (g/100 g).

10 Precision of the method

10.1 Interlaboratory test

Details of the test and the precision of the method are summarized in Annex C. The values derived from this interlaboratory test are not necessarily applicable to concentration ranges and matrices other than those given.

10.2 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, shall in not more than 5 % of cases exceed the value of *r* given in Tables C.1 to C.15.

10.3 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, shall in not more than 5 % of cases exceed the value of *R* given in Tables C.1 to C.15.

11 Test report

The test report shall contain at least the following information:

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

b) the sampling method used, if known;

c) the test method used, with reference to this document, i.e. ISO 24363:2023;

d) all operating details not specified in this document, or regarded as optional, together with details of any incidents that occurred when performing the method, which can have influenced the test result(s);

e) the test result obtained and the mode of expression (e.g. g/100 g);

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