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Animal and vegetable fats and oils — Enzymatic determination of total sterols content

Corps gras d'origines animale et végétale — Détermination enzymatique de la teneur en stérols totaux

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ISO copyright office
Ch. de Blandonnet 8 • CP 401
CH-1214 Vernier, Geneva, Switzerland
Tel. +41 22 749 01 11
Fax +41 22 749 09 47
copyright@iso.org
www.iso.org

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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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Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on 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 the following URL: www.iso.org/iso/foreword.html.

The committee responsible for this document is ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

This second edition cancels and replaces the first edition (ISO 11702:2009), of which it constitutes a minor revision. The scope has been revised to state that the document is not applicable to milk and milk fat products.

Animal and vegetable fats and oils — Enzymatic determination of total sterols content

1 Scope

This International Standard specifies a method for the quantitative determination of the total sterols content by means of an enzymatic staining test. The method is applicable to free and esterified sterols in animal and vegetable fats and oils, fatty foods and related products. The determination is applicable to sample quantities of 1 g to 2 g of fat.

The method is not applicable to dark coloured fats and oils. The enzyme is not specific for cholesterol, but also oxidizes other 3-hydroxysterols. The method has not been tested for products fortified with sterols at higher levels.

Milk and milk products (or fat coming from milk and milk products) are excluded from the scope of this International Standard.

NOTE The method is technically equivalent to IUPAC method 2.404[8] and DGF standard method F-III 2 (91).[7]

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.

ISO 661, *Animal and vegetable fats and oils — Preparation of test sample*

ISO 3696, *Water for analytical laboratory use — Specification and test methods*

3 Terms and definitions

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

3.1
total sterols content
$w_{sterols}$
mass fraction of sterols determined by the method specified in this International Standard

Note 1 to entry: For vegetable fats and oils, the sterols content is expressed as β-sitosterol; for animal fats, as cholesterol.

Note 2 to entry: The total sterols content is expressed in milligrams per 100 g of fat.

4 Principle

The test sample is saponified and the sterols in the unsaponifiable matter are determined enzymatically. Cholesterol is oxidized by cholesterol oxidase to cholestenone. The equimolar amount of hydrogen peroxide produced in the process oxidizes in the presence of catalase methanol to formaldehyde. In the presence of ammonium ions, with acetylacetone, it forms a yellow lutidine dye (3,5-diacetyl-1,4-dihydrolutidine). The latter is determined spectrophotometrically in the visible range at 405 nm. The concentration of the dye is equivalent to the amount of sterols.

NOTE Cholesterol oxidase oxidizes cholesterol and other sterols having a hydroxy group in the 3β-position. Therefore, phytosterols like stigmasterol and sitosterol are also determined.

5 Reagents

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

Unless otherwise stated, use only reagents of recognized analytical grade.

5.1 Water, complying with ISO 3696, grade 3 or better.

5.2 Isopropanol.

5.3 Acetone.

5.4 Acetylacetone.

5.5 Suspension of cholesterol oxidase,[1)] (EC 1.1.3.6) from *Nocardia erythropolis*, 15 U/ml.

5.6 Suspension of catalase, (hydrogen peroxide oxido-reductase)[1)] (EC 1.11.1.6) from bovine liver.

5.7 Hydrochloric acid, c(HCl) = 8 mol/l.

5.8 Methanolic potassium hydroxide solution, c(KOH) = 0,5 mol/l.

Dissolve 2,8 g potassium hydroxide in a small amount of hot methanol, cool, and dilute with methanol to 100 ml.

5.9 Ammonium phosphate buffer solution, adjusted to pH 7.

5.10 Solution 1.

Add 19,1 ml acetone (5.3) and 230 000 U catalase (5.6) to 50 ml buffer solution (5.9) in a 100 ml one-mark volumetric flask (6.4), and make up to the mark with water (5.1).

5.11 Solution 2.

Add 0,26 ml acetylacetone (5.4) and 1,10 ml acetone (5.3) to 25 ml water (5.1) in a 50 ml one-mark volumetric flask (6.4), and make up to the mark with water.

5.12 Solution 3.

Prior to use, mix three volumes of solution 1 (5.10) with two volumes of solution 2 (5.11).

NOTE Solution 3 can be kept in amber bottles for three months at 4 °C, provided it is prepared under sterile conditions.

6 Apparatus

6.1 Test tubes, of diameter 18 mm.

6.2 Filter funnel.

1) A suitable ready-made test kid for the colorimetric determination of cholesterol in foodstuffs and other materials is available from R-Biopharm. 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.

6.3 Fluted filter, suitable for the filter funnel (6.2).

6.4 One-mark volumetric flasks, of capacities 25 ml, 50 ml, and 100 ml, ISO 1042[2] class A.

6.5 Enzyme pipettes, of capacities 0,02 ml, ISO 7550,[6] to 1 ml, ISO 648[1] class A.

6.6 Pipette, of capacity 5 ml, ISO 648[1] class A.

6.7 Round bottomed flask, standard ground joint, of capacity 50 ml.

6.8 Test tubes with ground stoppers.

6.9 Spectrophotometer, set to 405 nm.

6.10 Glass cuvettes, pathlength 1 cm, suitable for the spectrophotometer (6.9).

6.11 Water bath, thermostatically controlled at 37 °C to 40 °C.

6.12 Refrigerator, capable of maintaining a temperature of 4 °C.

6.13 Glass beads.

6.14 Reflux condenser, standard ground joint.

7 Sampling

A representative sample should have been sent to the laboratory. It should not have 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.[3]

8 Preparation of the test sample

Prepare the test sample in accordance with ISO 661. Specific treatment of the test sample (filtration, melting, etc.) shall be mentioned in the test report.

9 Procedure

9.1 Saponification

9.1.1 Weigh 1 g to 2 g of the sample accurately to within 0,001 g into a 50 ml round bottomed flask (6.7). The sterol concentration in the test solution shall be between 0,02 g/l and 0,4 g/l. This requirement shall be taken into account during the weighing and diluting steps. In the case of saturated fats, the amount weighed shall be reduced, as otherwise, free fatty acids formed after saponification and acidification are not completely removed during filtration and affect the determination. Ensure at all times that the solution obtained is clear.

9.1.2 Add 10 ml of methanolic potassium hydroxide solution (5.8) and some glass beads (6.13). Heat the mixture and, when boiling, reflux for 25 min.

9.1.3 Transfer the still warm soap solution quantitatively into a 25 ml one-mark volumetric flask (6.4) and wash out the round bottomed flask (6.7) with a few millilitres of isopropanol (5.2).

9.1.4 Pipette (6.5) 1 ml of hydrochloric acid (5.7) into the 25 ml one-mark volumetric flask (6.4), make up to the mark with isopropanol (5.2) and shake vigorously. Ensure at all times that the solution obtained is clear.

9.1.5 Place the flask with the mixture (9.1.4) in the refrigerator (6.12) and maintain it at 4 °C for 20 min.

9.1.6 Next, filter the (turbid) solution as rapidly as possible through a fluted filter (6.3) and immediately use the filtrate for the enzymatic determination.

9.2 Enzymatic determination of the sterols content

9.2.1 Pipette (6.6) 5 ml of solution 3 (5.12) into a test tube (6.1) and add 0,4 ml of the filtrate (9.1.6). Mix thoroughly.

9.2.2 Transfer 2,5 ml of this mixture into a stoppered test tube (6.8) and add by pipette (6.5) 0,02 ml of the cholesterol oxidase suspension (5.5). Mix thoroughly.

9.2.3 Transfer the rest of the solution from 9.2.1 into another stoppered test tube (6.8) for use as the blank test.

9.2.4 Close the test tubes containing the sample and the blank, respectively, with the stoppers, and incubate them in the water bath (6.11) for 60 min at 37 °C to 40 °C.

9.2.5 After cooling to room temperature, immediately measure the extinctions of sample and blank against water (5.1), successively in the same cuvette, in the spectrophotometer (6.9) at 405 nm.

10 Result of the determination

The total sterols mass concentration, ρ, in grams per litre, of the sample filtrate, expressed as cholesterol for animal fats and as β-sitosterol for vegetable fats and oils, is calculated in accordance with Formula (1):

$$\rho = \frac{V_1 \; M}{\varepsilon \; l \; V_2 \times 1\,000} \Delta A \qquad (1)$$

where

V_1 is the volume, in millilitres, of the diluted filtrate (5,4 ml, see 9.2.1);

M is the molecular mass of cholesterol (M_{chol} = 386,64 g/mol) or β-sitosterol ($M_{\beta\text{-sito}}$ = 414,69 g/mol);

ε is the absorbance (extinction) coefficient of lutidine at 405 nm (7,4 l mmol^{-1} cm^{-1});

l is the pathlength, in centimetres, of the glass cuvette (1 cm);

V_2 is the volume, in millilitres, of the undiluted filtrate (0,4 ml, see 9.2.1);

ΔA is the difference between the absorbance of the blank test and that of the test portion, in which a dilution factor of 1,008 (2,52/2,50) needs to be taken into account:

$\Delta A = 1{,}008\ (A_1 - A_0)$

in which

A_1 is the absorbance of the test portion at 405 nm;

A_0 is the absorbance of the blank test at 405 nm.

The total sterols mass concentration, ρ, in grams per litre, of sample filtrate is then calculated using either Formula (2), for animal fats and oils:

$$\rho_{\text{chol}} = \frac{5,400 \times 386,64 \times 1,008}{7,4 \times 1,00 \times 0,400 \times 1\,000} \Delta A = 0,711\ \Delta A \tag{2}$$

or Formula (3), for vegetable fats and oils:

$$\rho_{\beta-\text{sito}} = \frac{5,400 \times 414,69 \times 1,008}{7,4 \times 1,00 \times 0,400 \times 1\,000} \Delta A = 0,763\ \Delta A \tag{3}$$

Considering the dilution (25 ml in 9.1.4), the total sterols content, w_{sterols}, of the sample, in milligrams per 100 g, is then calculated using Formula (4):

$$w_{\text{sterols}} = \frac{25 \times 100 \times 1\,000\ \rho}{1\,000\ m} \tag{4}$$

where m is the mass, in grams, of the test portion (9.1.1).

The total sterols mass fraction (whether on the cholesterol or β-sitosterol basis) is given as a whole number.

11 Precision of the method

11.1 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.

11.2 Repeatability limit

The repeatability limit, r, is the value less than or equal to which the absolute difference between two final values, each of them representing a series of test results obtained under repeatability conditions, is expected to be with a probability of 95 %.

Repeatability conditions are defined as conditions under which test results are obtained with the same method, on identical test material, in the same laboratory, by the same operator, using the same equipment and reagents, within a short interval of time.

11.3 Reproducibility limit

The reproducibility limit, R, is the value less than or equal to which the absolute difference between two final values, each of them representing a series of test results obtained under reproducibility conditions, is expected to be with a probability of 95 %.

Reproducibility conditions are defined as conditions under which test results are obtained with the same method, on identical test material, in different laboratories, by different operators, using different equipment and reagents, within a short interval of time.