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Animal and vegetable fats and oils — Determination of polyunsaturated fatty acids with a *cis,cis* 1,4-diene structure

Corps gras d'origines animale et végétale — Dosage des acides gras polyinsaturés ayant une configuration cis-cis *diénique-1,4*

Reference number
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Foreword

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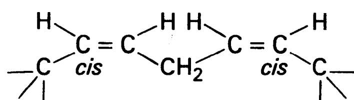
Animal and vegetable fats and oils — Determination of polyunsaturated fatty acids with a *cis,cis* 1,4-diene structure

1 Scope and field of application

This International Standard specifies an enzymic method for the determination in animal and vegetable fats and oils of polyunsaturated fatty acids with a *cis,cis* 1,4-diene structure, in practice those of the linoleic (9,12-octadecadienoic) and linolenic (9,12,15-octadecatrienoic) acid series having ω3 and ω6 unsaturation. The structure is



It is not applicable to fats and oils containing polyunsaturated fatty acids of the ω8 and ω9 series or containing branched chain fatty acids.

2 References

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

ISO 5555, *Animal and vegetable fats and oils — Sampling.*

3 Definition

For the purposes of this International Standard, the following definition applies.

cis,cis **1,4-diene fatty acids** : Fatty acids determined by the procedure specified in this International Standard.

They are expressed as a percentage by mass of the sample.

4 Principle

Saponification of a test portion at ambient temperature followed by liberation of the fatty acids. Enzymic oxidation of the fatty acids containing a *cis,cis* 1,4-diene structure. Measurement, at the wavelength of maximum absorbance (about 235 nm) of the absorbance due to these oxidized acids, while compensating for the absorbance due to any dienoic conjugated acids initially present in the sample.

5 Reagents and materials

All reagents shall be of recognized analytical quality, and the water used shall be distilled water or water of equivalent purity.

5.1 ***n*-Hexane.**

5.2 Hydrochloric acid, solution, $c(HCl) \approx 0,5$ mol/l.

5.3 Potassium hydroxide, solution in ethanol, $c(KOH) \approx 0,5$ mol/l.

5.3.1 Stock solution

Dissolve 65 g of potassium hydroxide (86 % KOH) in about 80 ml of water. Cool and make up to 100 ml.

5.3.2 Preparation

Dilute 5 ml of the stock solution (5.3.1) to 100 ml with 95 % (V/V) ethanol.

This solution shall be freshly prepared.

5.4 Potassium borate, buffer solution, $c(K_3BO_3) = 1,0$ mol/l (pH = 9,0).

Dissolve 61,9 g of boric acid (H_3BO_3) and 25,0 g of potassium hydroxide (86 % KOH) in about 800 ml of water with heating and stirring. Allow to cool to room temperature, then check the pH and, if necessary, adjust the pH to 9,0 with hydrochloric acid or potassium hydroxide solution. Dilute to 1 000 ml with water.

5.5 Potassium borate, buffer solution, $c(K_3BO_3) = 0,2$ mol/l (pH = 9,0).

Dilute 200 ml of 1,0 mol/l potassium borate buffer solution (5.4) to 1 000 ml with water. Cool on ice.

5.6 Lipoxidase, dilute solution.

5.6.1 Lipoxidase, with an activity of at least 50 000 units per milligram (1 unit of activity is defined as the amount of enzyme which oxidizes $1,2 \times 10^{-4}$ µmol of linoleic acid per minute under the conditions of the test).

In the freeze-dried state, the enzyme is stable for several years when kept at a temperature of − 18 °C or below.

NOTE — Enzyme preparations of low specific activity may give rise to erroneously low results. Preparations of very high specific activity give no better results than those of activities from 50 000 to 100 000 units per milligram.

5.6.2 Stock solution

Dissolve an amount of the enzyme (5.6.1) solution equivalent to about 650 000 units of activity in 10 ml of ice-cold 0,2 mol/l potassium borate buffer solution (5.5).

The stock solution may be kept at − 18 °C or below for a considerable length of time.

5.6.3 Preparation

Mix 2 ml of the stock solution (5.6.2) with 8 ml of ice-cold 0,2 mol/l potassium borate buffer solution (5.5).

5.7 Lipoxidase, inactivated solution.

Transfer a few millilitres of the dilute lipoxidase solution (5.6) to a test-tube, ensuring that no droplets of the solution adhere to the walls of the test-tube. Immerse the test-tube in a boiling water-bath (6.6) for at least 5 min, keeping the surface of the solution well below the surface of the water-bath.

5.8 Reference oil, for example a sunflower or cottonseed oil, of known polyunsaturated fatty acids content (determined accurately by gas-liquid chromatography and expressed as a percentage by mass of the reference oil) for which the polyunsaturated fatty acids are assumed to consist entirely of fatty acids with a *cis,cis* 1,4-diene structure.

NOTE — Trilinolein, free from geometrical and chiral isomers, may be used as a reference.

5.9 Nitrogen, purity 99,5 % (m/m) minimum.

6 Apparatus

All glassware shall be scrupulously clean.

Usual laboratory equipment and in particular

6.1 Volumetric flasks, stoppered, of capacity 100 ml.

6.2 Pipettes, of capacities 1, 10, and 20 ml.

6.3 Graduated pipettes, of capacities 1 and 10 ml.

6.4 Test-tubes, stoppered, of capacity 10 ml, completely dry. Alternatively, (see note 1 to 9.6.2) spectrometer cells (see 6.8) fitted with stoppers.

6.5 Centrifuge, with 10 ml centrifuge tubes.

6.6 Water-bath, boiling.

6.7 Water-bath, capable of being maintained at 50 ± 2 °C.

6.8 Spectrometer, capable of measuring absorbance at about 235 nm, equipped with silica cells of thickness 10 mm.

6.9 Analytical balance.

7 Sampling

See ISO 5555.

8 Preparation of test sample

Prepare the test sample in accordance with ISO 661.

9 Procedure

9.1 Check test

It is recommended that a sample of reference oil with an accurately known content of polyunsaturated fatty acids (5.8) be analysed in parallel with the test sample, in order to check the procedure.

9.2 Test portion

Weigh, to the nearest 0,1 mg, 50 to 200 mg of the test sample (clause 8) (depending on the expected amount of polyunsaturated fatty acids) into a 100 ml volumetric flask labelled A (6.1).

NOTE — When the amount of sample taken is equivalent to 10 to 80 mg of polyunsaturated fatty acids, the measured absorbance at the maximum will be in the range 0,07 to 0,5.

9.3 Saponification

Transfer, by means of a pipette (6.2), 10 ml of ethanolic potassium hydroxide solution (5.3) to the flask and displace the air in the flask with nitrogen (5.9). Stopper the flask and store it in the dark, allowing the saponification to proceed for at least 4 h, with occasional shaking of the flask to mix the contents.

If the sample has a melting point above room temperature, it is advisable to warm the flask and contents (after stoppering) for a few minutes in a water-bath (6.7) at 50 ± 2 °C in order to speed up the saponification.

NOTE — An alternative saponification procedure which is particularly suitable for replicate analysis of multiple samples is specified in the annex.

9.4 Preparation of the test solution

After saponification is completed, add, using the appropriate pipettes (6.2), 20 ml of the 1,0 mol/l potassium borate buffer solution (5.4) and 10 ml of the hydrochloric acid (5.2) to flask A. Make up to the 100 ml mark with water. Stopper the flask and mix the contents by gently inverting the flask a few times, keeping foaming to a minimum. If necessary, readjust the volume to 100 ml after mixing. If a precipitate is formed at this stage, transfer a few millilitres of the mixed solution to a centrifuge tube (6.5) and spin down the precipitate.

Transfer by means of a pipette (6.2), 1 ml of the contents of the flask A (or 1 ml of the supernatant liquid from the centrifuged solution) into another 100 ml flask (6.1) labelled B, previously flushed with nitrogen (5.9). When the sample is expected to contain very low amounts of polyunsaturated fatty acids, transfer 2 to 4 ml to the flask B rather than 1 ml.

Transfer, by means of a pipette (6.2), 20 ml of 1,0 mol/l potassium borate buffer solution (5.4) to flask B and make up to the 100 ml mark with water. Stopper the flask and mix the contents, keeping foaming to a minimum (see the note). A slight turbidity at this stage will not interfere with the subsequent measurements.

NOTE — After saponification a dilute solution of soap is obtained. The concentration of the soap in the flask A is about 1 mg/ml, and in the flask B is about 10 µg/ml. The concentration of soaps is higher in the foam than in the bulk of the solution. If foam is adhering to the pipette when the solution is transferred from one flask to another, this may cause transference of an unknown excess of fatty acids.

9.5 Matching test

Carry out a matching test in parallel with the determination (9.7), using the same procedure, but using 0,1 ml of inactivated lipoxidase solution (5.7) instead of the dilute lipoxidase solution (5.6) to prepare a sample compensation solution.

9.6 Calibration

9.6.1 Preparation of the set of calibration solutions

Weigh, to the nearest 0,1 mg, an amount of the reference oil equivalent to about 100 mg of polyunsaturated fatty acids into a 100 ml volumetric flask (6.1). Saponify and make up to the mark as specified in 9.3 and the first paragraph of 9.4.

Transfer 10 ml of the solution to a second 100 ml volumetric flask (6.1), add 18 ml of 1,0 mol/l potassium borate buffer solution (5.4) using a graduated pipette (6.3) and make up to the 100 ml mark with water.

Transfer, by means of a graduated pipette (6.3), 1, 2, 4, 6, 8 and 10 ml volumes from this second flask to a series of six 100 ml volumetric flasks (6.1) and make up to the 100 ml mark with 0,2 mol/l potassium borate buffer solution (5.5).

9.6.2 Enzymic oxidation

Transfer, by means of a 1 ml graduated pipette (6.3), 0,1 ml of dilute lipoxidase solution (5.6) to a series of six test-tubes (6.4) (see note 1). Then add 3 ml of each calibration solution to each of the test-tubes (one dilution per test-tube) and shake gently to ensure that the solutions are mixed (see note 2).

Allow the tubes to stand for 20 to 30 min.

NOTES

1 The oxidation procedure may be carried out in a stoppered spectrometer cell (see 6.8) in order to avoid the need to transfer the solution to the cell prior to measurement of the absorbance.

2 The handling of the contents of the test-tube is important. After the initial mixing of the contents, no further mixing should be done. Further mixing results in increased absorbance in the calibration solutions, the sample compensation solution and the test solution. Also it is generally not possible to check if a measured value has been read correctly if the solution has been emptied from the cell and subsequently replaced. The reason for the increase in absorbance on handling of the solutions is not understood. Consequently each laboratory should adhere to a fixed procedure.

9.6.3 Spectrometric measurements

Transfer the contents of each test-tube to individual silica cells (see 6.8). Using the spectrometer (6.8), measure the absorbance of each of the calibration solutions at the wavelength of maximum absorbance (approximately 235 nm), using the sample compensation solution (see 9.5) for zero adjustment of the instrument. Take the mean of two absorbance readings for each calibration solution.

9.6.4 Plotting the calibration graph

Plot the mean values of the absorbances against the masses of polyunsaturated fatty acids calculated from the known composition of the reference oil.

Draw the best straight line through the points plotted; this straight line shall pass through the origin.

9.7 Determination

9.7.1 Enzymic oxidation

Transfer, by means of a 1 ml graduated pipette (6.3), 0,1 ml of dilute lipoxidase solution (5.6) to a test-tube (6.4) (see note 1 to 9.6.2). Then add 3 ml of the test solution (9.4) from flask B to the test-tube and shake gently to ensure that the solution is mixed (see note 2 to 9.6.2).

Allow the tube to stand for 20 to 30 min.

9.7.2 Spectrometric measurements

Transfer the contents of the tube to a silica cell (see 6.8). Using the spectrometer (6.8), measure the absorbance of the test solution at the wavelength of maximum absorbance (approximately 235 nm), using the sample compensation solution (see 9.5) for zero adjustment of the instrument. Take the mean of two absorbance readings and read the mass of fatty acids from the calibration graph (9.6.4).

NOTE — The zero adjustment using the sample compensation solution compensates for the absorbance due to any dienoic conjugated acids initially present in the sample. The absorbance value of the sample compensation solution should be checked against water because if it is too high compared with the test solution, precision will be reduced.

9.8 Number of determinations

Carry out two determinations on the same test sample.

10 Expression of results

10.1 Method of calculation

The polyunsaturated fatty acids content, expressed as a percentage by mass, is equal to

$$\frac{m_1 \times 100}{V m_0}$$

where

m_0 is the mass, in milligrams, of the test portion;

m_1 is the mass, in milligrams, of polyunsaturated fatty acids read from the calibration graph;

V is the numerical value of the volume, in millilitres, of solution taken from the flask A (usually 1 ml).

NOTE — The result obtained in this way is expressed on the whole fat or oil basis and not on the total of the fatty acids in the fat or oil.

10.2 Repeatability

The difference between the results of two determinations carried out in rapid succession by the same analyst, under the same conditions on the same test sample, shall not exceed 3,5 % (m/m) (absolute value) of polyunsaturated fatty acids in the range 10 to 70 % (m/m).

11 Test report

The test report shall show the method used and the result obtained, indicating clearly the method of expression used. It shall also mention any operating details not specified in this International Standard, or regarded as optional, together with details of any incidents likely to have influenced the result.

The test report shall include all the information necessary for the complete identification of the sample.

Annex

Alternative procedure for saponification

(This annex forms an integral part of the standard.)

Dissolve the test portion (9.2) in a few millilitres of *n*-hexane (5.1), then dilute to the mark with the same solvent and mix. Transfer, by means of a pipette (6.2), 1,0 ml of the solution to a 100 ml one-mark volumetric flask (6.1) labelled B, previously flushed with nitrogen. When the sample is expected to contain very low amounts of polyunsaturated fatty acids, transfer 2 to 4 ml rather than 1,0 ml to flask B. Completely evaporate the solvent under a gentle stream of nitrogen.

To the solvent-free sample in flask B add 2 ml of ethanolic potassium hydroxide solution (5.3) and stopper the flask. Leave the flask in the dark and allow saponification to take place for at least 4 h. Proceed as in 9.7.

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