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**Animal and vegetable fats and oils —  
Determination of the composition of fatty  
acids in the 2-position of the triglyceride  
molecules**

*Corps gras d'origines animale et végétale — Détermination de la  
composition des acides gras en position 2 dans les triglycérides*



## 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 6800 was prepared by Technical Committee ISO/TC 34, *Agricultural food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

This second edition cancels and replaces the first edition (ISO 6800:1985), which has been technically revised.

Annexes A to C of this International Standard are for information only.

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International Organization for Standardization  
Case postale 56 • CH-1211 Genève 20 • Switzerland  
Internet central@iso.ch  
X.400 c=ch; a=400net; p=iso; o=isocs; s=central

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# Animal and vegetable fats and oils — Determination of the composition of fatty acids in the 2-position of the triglyceride molecules

## 1 Scope

This International Standard specifies a method for the determination of the composition of fatty acids which are esterified in the 2-position ( $\beta$  or internal position) of the triglyceride molecules in animal and vegetable fats and oils.

Because of the nature of pancreatic lipase action, the method is applicable only to fats and oils with a melting point below 45 °C.

The method is not unreservedly applicable to all fats and oils, particularly those containing substantial amounts of

- fatty acids with 12 or fewer carbon atoms (e.g. copra oil, palm kernel oil, butyric butter fats);
- fatty acids with 20 and more carbon atoms and of a high degree of unsaturation (more than four double bonds) (e.g. fish oil and marine animal oil);
- fatty acids which have secondary groups containing oxygen.

NOTE — Fatty acids with double bonds in the ( $n$ -16) to ( $n$ -11) position (e.g. petroselinic acid) are converted only very slowly by pancreatic lipase. This may lead to wrong results.

## 2 Normative references

The following standards contain provisions which, through reference in this text, constitute provisions of this International Standard. At the time of publication, the editions indicated were valid. All standards are subject to revision, and parties to agreements based on this International Standard are encouraged to investigate the possibility of applying the most recent editions of the standards indicated below. Members of IEC and ISO maintain registers of currently valid International Standards.

ISO 660:1996, *Animal and vegetable fats and oils – Determination of acid value and of acidity.*

ISO 661:1989, *Animal and vegetable fats and oils – Preparation of test sample.*

ISO 3696:1987, *Water for analytical laboratory use – Specification and test methods.*

ISO 5508:1990, *Animal and vegetable fats and oils – Analysis by gas chromatography of methyl esters of fatty acids.*

ISO 5509:—<sup>1)</sup> *Animal and vegetable fats and oils – Preparation of methyl esters of fatty acids.*

### 3 Principle

After neutralization, where necessary, of any free fatty acids, purification of the test portion by column chromatography. Partial enzymatic hydrolysis of the glycerides to yield 2-monoglycerides. Separation of the monoglycerides by thin-layer chromatography (TLC) and determination of their fatty acid composition by gas chromatography.

### 4 Reagents

Use only reagents of recognized analytical grade and water in accordance with grade 2 of ISO 3696.

#### 4.1 Reagents for the purification of the test portion

**4.1.1 2-Propanol**, or **ethanol**, 95 % (V/V).

**4.1.2 Hexane** (if available) or **light petroleum** (boiling range 30 °C to 60 °C).

**4.1.3 2-Propanol**, 50 % (V/V), or **ethanol**, 50 % (V/V).

**4.1.4 Sodium hydroxide**, 0,5 mol/l solution.

**4.1.5 Phenolphthalein solution**, 1 g per 100 ml of ethanol, 95 % (V/V).

**4.1.6 Activated neutral alumina**, for chromatography, Brockmann activity I, recently activated for 2 h at 260 °C and kept in a desiccator.

**4.1.7 Nitrogen.**

#### 4.2 Reagents for hydrolysis of the triglycerides

**4.2.1 Diethyl ether**, free from peroxides.

**4.2.2 Hydrochloric acid**, 6 mol/l solution.

**4.2.3 Sodium cholate**, 1 g/l solution of enzymatic quality.

**4.2.4 Calcium chloride**, 220 g/l solution.

**4.2.5 Buffer solution**, 2-amino-2-(hydroxymethyl)propan-1,3-diol<sup>2)</sup>, 1 mol/l, adjusted to pH 8 with hydrochloric acid (4.2.2) using a pH-meter.

Store this solution at between 0 °C and 4 °C and use within 14 days.

1) To be published. (Revision of ISO 5509:1978)

2) Alternative names are: tris(hydroxymethyl)methylamine; tris(hydroxymethyl)aminomethane.

**4.2.6 Pancreatic lipase**, with an activity of between 8 and 20 units/mg.

Store dry in a refrigerator. Before use, bring a portion of the powder to ambient temperature.

NOTE — Lipase of suitable activity is available commercially. If preferred, the lipase may be prepared and assayed in accordance with the procedure described in annex A.

**4.3 Reagents for the isolation of the 2-monoglycerides****4.3.1 Ethanol**, 95 % (V/V).**4.3.2 Hexane** (if available) or **light petroleum**, boiling range 30 °C to 60 °C.**4.3.3 Acetone**.**4.3.4 Silica powder**, with binder, for thin-layer chromatography.**4.3.5 Developing solvent**, prepared as follows:

|                                           |       |
|-------------------------------------------|-------|
| hexane (if available) or light petroleum: | 70 ml |
| diethyl ether:                            | 30 ml |
| formic acid, 98 % (V/V) min.:             | 1 ml  |

**4.3.6 2',7'-Dichlorofluorescein**, indicator solution, 2 g/l in ethanol, rendered slightly alkaline by addition of a drop of 1 mol/l sodium hydroxide per 100 ml of the solution.**4.4 Reagents for the analysis of the 2-monoglycerides by gas chromatography**

See ISO 5508 and ISO 5509.

**5 Apparatus**

Usual laboratory equipment and, in particular, the following.

**5.1 Apparatus for the purification of the test portion****5.1.1 Water bath**, thermostatically controlled, and capable of being maintained at 30 °C to 40 °C.**5.1.2 Glass column**, for chromatography, 13 mm internal diameter and 400 mm in length, equipped with a sintered glass plate and a tap.**5.1.3 Rotary evaporator**, with 250 ml flask.**5.1.4 Tubing**, for bubbling nitrogen.**5.1.5 Separating funnel**, of 500 ml capacity.**5.1.6 Round-bottom flask**, of 100 ml capacity.

## 5.2 Apparatus for the hydrolysis of the triglycerides

### 5.2.1 Centrifuge.

5.2.2 **Centrifuge tube**, glass, of 10 ml capacity, with ground stopper.

5.2.3 **Vibrating electric shaker**, for vigorous agitation of the centrifuge tube.

5.2.4 **Water bath**, thermostatically controlled, and capable of being maintained at  $40\text{ }^{\circ}\text{C} \pm 0,5\text{ }^{\circ}\text{C}$ .

5.2.5 **Hypodermic syringe**, of 1 ml capacity, with thin needle.

5.2.6 **Stopwatch**.

## 5.3 Apparatus for the isolation of the 2-monoglycerides

5.3.1 **Developing tank**, for thin-layer chromatography, with ground glass lid, suitable for containing glass plates 200 mm x 200 mm.

5.3.2 **Spreader and rack**, for preparation of the plates.

5.3.3 **Glass plates**, 200 mm x 200 mm.

5.3.4 **Microsyringe**, capable of dispensing drops of 3  $\mu\text{l}$  to 4  $\mu\text{l}$ .

5.3.5 **Apparatus for spraying the indicator solution onto the plates**

5.3.6 **Microspatula**.

5.3.7 **Oven**, capable of being maintained at  $103\text{ }^{\circ}\text{C} \pm 2\text{ }^{\circ}\text{C}$ .

5.3.8 **Ultraviolet lamp**, for examining chromatographic plates, for example with a wavelength of 254 nm.

5.3.9 **Round-bottom flask**, of 25 ml capacity, with **air condenser** of approximately 1 m length, with ground joint.

5.3.10 **Conical flask**, of 250 ml capacity, with ground stopper.

5.3.11 **Conical flask**, of 50 ml capacity (if necessary).

5.3.12 **Filter**, of sintered glass, porosity P 40 (16  $\mu\text{m}$  to 40  $\mu\text{m}$ ) (if necessary).

5.3.13 **Desiccator**, containing an efficient desiccant.

## 5.4 Apparatus for the analysis of the 2-monoglycerides by gas chromatography

See ISO 5508 and ISO 5509.

## 6 Sampling

It is important that the laboratory receive a sample which is truly representative and has not been damaged or changed during transport and storage.

Sampling is not part of the method specified in this International Standard. A recommended sampling method is given in ISO 5555 [1].

## 7 Preparation of test sample

Prepare the test sample from the laboratory sample in accordance with ISO 661.

## 8 Procedure

NOTE — If it is required to check whether the repeatability requirement (see 10.2) is met, carry out two single determinations in accordance with 8.1 to 8.6.

### 8.1 Determination of the acidity of the test sample

Determine the acidity of the test sample in accordance with ISO 660.

If the acidity is below 3 % (*m/m*), purify the sample through alumina in accordance with 8.3.

If the acidity exceeds 3 % (*m/m*), first neutralize the sample with sodium hydroxide in the presence of solvent in accordance with 8.2 then purify through alumina in accordance with 8.3.

### 8.2 Neutralization with sodium hydroxide

Dissolve about 10 g of the test sample in 100 ml of hexane (if available) or light petroleum (4.1.2) and transfer the solution to the separating funnel (5.1.5). Add 50 ml of 2-propanol or ethanol (4.1.1), a few drops of phenolphthalein solution (4.1.5) and a volume of sodium hydroxide solution (4.1.4) equivalent to the free acidity of the fat or oil, with an excess of 0,5 %. Shake vigorously for 1 min, add 50 ml of water, shake again and allow to stand. When separated, run off the lower layer containing the soap and any intermediate layers (mucilages and insoluble substances). Wash the hexane or light petroleum solution of the neutralized oil with successive 25 ml or 30 ml portions of a solution of 2-propanol or ethanol (4.1.3) until the pink colour of the phenolphthalein disappears.

Transfer the solution to the rotary evaporator flask (5.1.3) and remove most of the solvent by evaporating under reduced pressure. Dry the oil at 30 °C to 40 °C under reduced pressure using a nitrogen stream (4.1.7) until the solvent is completely removed.

### 8.3 Purification of the test portion through alumina

Prepare a suspension of 15 g of activated alumina (4.1.6) in 50 ml of hexane (if available) or light petroleum (4.1.2) and pour, while shaking, into a glass column for chromatography (5.1.2). Ensure that the alumina settles evenly and allow the solvent level to fall to 1 mm to 2 mm above the upper level of the adsorbent. Carefully pour into the column a solution prepared by dissolving a test portion of 5 g, neutralized if necessary, in 25 ml of hexane (if available) or light petroleum (4.1.2) and collect all the liquid which elutes from the column in a 100 ml round-bottom flask (5.1.6).

Remove most of the solvent by evaporation under reduced pressure, then dry the oil at 30 °C to 40 °C using a nitrogen stream (4.1.7) until the solvent is completely removed.

## 8.4 Hydrolysis of the triglycerides

**8.4.1** Weigh approximately 0,1 g of the purified test portion (8.3) in a 10 ml centrifuge tube (5.2.2). If this test portion is not liquid at ambient temperature, place the tube in a water bath set at between 60 °C and 65 °C. If by then the test portion is not completely liquid, continue the immersion for not more than another 10 s.

Remove the tube from the water bath and continue directly and in rapid succession with the procedure steps 8.4.2 to 8.4.5.

**8.4.2** Add to the liquefied test portion a previously weighed amount of 20 mg of lipase (4.2.6) and add 2 ml of the buffer solution (4.2.5). Shake carefully and add successively 0,5 ml of sodium cholate solution (4.2.3) and 0,2 ml of calcium chloride solution (4.2.4).

Insert the stopper, shake carefully and place the tube immediately in the water bath set at 40 °C, maintaining manual shaking for  $60 \text{ s} \pm 2 \text{ s}$ . These actions shall be executed within approximately 30 s.

**8.4.3** Remove the tube from the water bath and shake vigorously at 40 °C for  $120 \text{ s} \pm 2 \text{ s}$  using the shaker (5.2.3).

**8.4.4** Immediately add 1 ml of hydrochloric acid (4.2.2) and approximately 1 ml of diethyl ether (4.2.1). Insert the stopper and shake vigorously using the shaker (5.2.3).

**8.4.5** Centrifuge and transfer the organic phase to a test tube using the syringe (5.2.5). If the test portion was solid at ambient temperature, repeat the extraction with a further 1 ml portion of diethyl ether and combine the extracts in the test tube.

## 8.5 Isolation of the 2-monoglycerides

### 8.5.1 Preparation of the plates

NOTE — Fully prepared plates are available commercially.

Carefully clean the glass plates (5.3.3) with ethanol (4.3.1), hexane or light petroleum (4.3.2) and acetone (4.3.3) until any fatty matter is completely removed.

Weigh 30 g of silica (4.3.4) into a 250 ml conical flask (5.3.10). Add 60 ml of water. Insert the stopper and shake vigorously for 1 min. Immediately introduce the slurry into the spreader (5.3.2). Spread a layer 0,25 mm thick on the clean plates. Allow the plates to dry for at least 1 h in air.

In all cases, whether the plates have been prepared as above or commercially prepared, activate the plates in the oven (5.3.7) set at 103 °C for 1 h. Before use, allow the plates to cool to ambient temperature in the desiccator (5.3.13).

In order to avoid acyl migrations, the silica gel plates shall be impregnated with boric acid. The reaction mixture shall be separated by TLC as soon as possible.

As certain silicas contain organic products which may affect the fatty acids during the analysis by chromatography, it is recommended that a blank test be carried out to ensure that there are no such substances present. Otherwise, clean the prepared plates beforehand by placing them in a developing tank with the solvent (4.3.5) and allowing the solvent to reach the top of the plate.

### 8.5.2 Separation of the 2-monoglycerides

Using a microsyringe (5.3.4), transfer the extract (8.4.5) onto a prepared plate (8.5.1) as a continuous band of fine droplets 15 mm from one of the edges.

Stand the plate in the developing tank (5.3.1), which has previously been saturated with the developing solvent (4.3.5). Replace the lid and develop the plate until the front of the solvent reaches a point 10 mm from the upper edge.

Develop the plate at a temperature of approximately 20 °C.

Dry the plate in air at approximately 20 °C and spray it with the indicator solution (4.3.6) using the apparatus (5.3.5). Mark the band of monoglycerides ( $R_f = 0,035$  approximately) under ultraviolet light (5.3.8) and scrape off using a microspatula (5.3.6). Avoid removing the components which remain on the starting line.

For purified test portions (8.3) which were liquid at ambient temperature, transfer the collected silica to a 25 ml methylation flask (5.3.9) and proceed as described in 8.6.

For purified test portions (8.3) which were solid at ambient temperature, transfer the silica to a 50 ml conical flask (5.3.11) with 15 ml of diethyl ether. Shake vigorously and transfer all the silica to a sintered glass filter (5.3.12). Wash the filter three times, each time with a 15 ml portion of diethyl ether, and collect the filtrate in an evaporator flask. Evaporate the diethyl ether solution down to between 4 ml and 5 ml and transfer it to a previously weighed 25 ml round-bottom flask (5.3.9). Then evaporate the solvent in a stream of nitrogen. Weigh the residue. The amount of monoglycerides obtained should be between 10 % ( $m/m$ ) and 30 % ( $m/m$ ) of the test portion. If this is not the case, repeat the hydrolysis of the triglycerides (8.3) or recheck the activity of the lipase (see annex A).

#### **8.6 Analysis of the 2-monoglycerides by gas chromatography**

Prepare the methyl esters of the fatty acids in the monoglycerides by treating the collected silica (or the monoglycerides extracted from the silica) directly in accordance with ISO 5509, using the general boron trifluoride method or the alternative method applicable to neutral fats and oils. Proceed with the gas chromatography of the methyl esters in accordance with ISO 5508.

### **9 Expression of results**

Calculate the proportions of the 2-position fatty acid esters, expressed as a percentage by mass of the total fatty acid esters of the 2-monoglycerides.

Give the results to one decimal place.

### **10 Precision**

#### **10.1 Interlaboratory tests**

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

#### **10.2 Repeatability**

The absolute difference between two independent single test results, obtained using 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, should not exceed:

0,2 % ( $m/m$ ) for constituent contents < 5 % ( $m/m$ );

1 % ( $m/m$ ) or 3 % of the mean of the two results for constituent contents  $\geq$  5 % ( $m/m$ ).

### 10.3 Reproducibility

The absolute difference between two single test results, obtained using the same method on identical test material in different laboratories with different operators using different equipment, should not exceed:

0,5 % (m/m) for constituent contents < 5 % (m/m);

3 % (m/m) or 10 % of the mean of the two results for constituent contents  $\geq$  5 % (m/m).

## 11 Test report

The test report shall specify

- the method in accordance with which sampling was carried out (if known),
- the method used,
- the test result(s) obtained, and
- if the repeatability has been checked, the final quoted result obtained.

It shall also mention any operating conditions not specified in this International Standard, or regarded as optional, together with details of any incidents likely to have influenced the results.

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

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## Annex A (normative)

### Preparation of the lipase and checking its activity

#### A.1 Preparation of the lipase

Cool 5 kg of fresh pork pancreas to 0°C. Remove the solid fat and connective tissue surrounding it and macerate the pancreas until a liquid paste is obtained. Stir this paste for 4 h to 6 h with 2,5 litres of dry acetone in the cold and then centrifuge.

Extract the residue three more times using the same volume of acetone, twice using a mixture of one part by volume of acetone to one part by volume of diethyl ether, and twice with diethyl ether.

Dry the residue for 48 h under reduced pressure until a stable powder is obtained. Store this powder in a refrigerator.

#### A.2 Checking of the lipase activity

Prepare an oil emulsion by shaking a mixture of 165 ml of a 100 g/l solution of gum arabic, 15 g of crushed ice and 20 ml of a previously neutralized oil for approximately 10 min in a suitable mixer.

Place 10 ml of the emulsion, 0,3 ml of sodium chlorate solution (200 g/l) and 20 ml of distilled water successively into a 50 ml beaker.

Place the beaker in a water bath set at 37 °C (see note 1).

Insert the electrodes of a pH-meter and a propeller stirrer into the beaker and, using a 5 ml burette, add a solution of 0,1 mol/l sodium hydroxide drop by drop until a pH of 8,5 is reached.

Add an adequate (see note 2) and accurately measured volume of a 0,1 % (*m/m*) aqueous suspension of the lipase powder under assay. As soon as the pH-meter shows the pH to be 8,3 start a stopwatch and add 0,1 mol/l sodium hydroxide solution so that the pH value of 8,3 is maintained. Note the volume of alkaline solution used every minute for about 10 min.

Plot the data obtained on a graph, using the x-axis for the time and the y-axis for the volume, in millilitres, of alkaline solution used to maintain the constant pH. The result should be a straight line.

#### NOTES

1 As a liquid oil is used, the hydrolysis temperature is fixed at 37 °C. However for the test, it is fixed at 40 °C so that fats with a melting point below 45 °C can be examined.

2 For the purposes of the test, use a quantity of lipase suspension which results in 1 ml of the alkaline solution being consumed in approximately 4 min or 5 min. Such a result is usually obtained by using 2 ml to 5 ml of lipase suspension, i.e. 1 mg to 5 mg of powder.

The lipase unit is defined as the quantity of enzyme which is required to release 1 µmol of acid per minute at 37 °C and pH 8,3.

The activity, *A*, expressed in lipase units per milligram, of the powder used, is given by the equation

$$A = \frac{V \cdot c}{m}$$

where

- $V$  is the volume of sodium hydroxide solution consumed, divided by time, calculated from the graph, in millilitres per minute;
- $m$  is the mass in milligrams of the test portion of lipase powder; the lipase used should have an activity of between 8 and 20 lipase units per milligram;
- $c$  is the concentration of sodium hydroxide solution, in millimoles per litre ( $c = 100$  mmol/l).

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