International Standard 6800

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

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

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Foreword

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Users should note that all International Standards undergo revision from time to time and that any reference made herein to any other International Standard implies its latest edition, unless otherwise stated.

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

1 Scope

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

2 Field of application

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 (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) (fish oil and marine animal oil);

— fatty acids which have secondary groups containing oxygen.

3 References

ISO 660, *Animal and vegetable fats and oils — Determination of acid value and of acidity.*

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

ISO 5508, *Animal and vegetable fats and oils — Analysis by gas-liquid chromatography of methyl esters of fatty acids.*

ISO 5509, *Animal and vegetable fats and oils — Preparation of methyl esters of fatty acids.*

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

4 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 and determination of their fatty acid composition by gas chromatography.

5 Reagents

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

5.1 Reagents for the purification of the test portion

5.1.1 2-propanol, or **ethanol** 95 % (V/V).

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

5.1.3 2-propanol 50 % (V/V) or **ethanol** 50 % (V/V).

5.1.4 Sodium hydroxide solution, 0,5 mol/l.

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

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

5.1.7 Nitrogen.

5.2 Reagents for hydrolysis of the triglycerides

5.2.1 Diethyl ether, free from peroxides.

5.2.2 Hydrochloric acid, 6 mol/l.

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

5.2.4 Calcium chloride ($CaCl_2$) solution, 220 g/l.

5.2.5 Buffer solution, 2-amino-2-(hydroxymethyl) propane-1,3-diol[1], 1 mol/l, adjusted to pH 8 with 6 mol/l hydrochloric acid (5.2.2) using a pH-meter.

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

5.2.6 Pancreatic lipase, with an activity of between 8 and 20 units/mg (see the annex).

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 the annex.

5.3 Reagents for the isolation of the 2-monoglycerides

5.3.1 Ethanol.

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

5.3.3 Acetone.

5.3.4 Silica powder, with binder, for thin layer chromatography.

5.3.5 Developing solvent, prepared as follows :

hexane (if available) or light petroleum :	70 ml
diethyl ether :	30 ml
formic acid, minimum 98 % (*V*/*V*) :	1 ml

5.3.6 2', 7'-Dichlorofluorescein, indicator solution, 2 g per litre of methanol, rendered slightly alkaline by addition of a drop of 1 mol/l sodium hydroxide per 100 ml of the solution.

5.4 Reagents for the analysis of the 2-monoglycerides by gas chromatography

See ISO 5508 and ISO 5509.

6 Apparatus

Usual laboratory equipment, and in particular

6.1 Apparatus for the purification of the test portion

6.1.1 Water-bath, thermostatically controlled, and capable of being maintained at 30 to 40 °C.

6.1.2 Glass column, for chromatography, 13 mm internal diameter and 400 mm in length, equipped with a sintered glass plate and a tap.

6.1.3 Rotary evaporator, with 250 ml flask.

6.1.4 Tubing, for nitrogen bubbling.

6.1.5 Separating funnel, 500 ml capacity.

6.1.6 Round-bottom flask, 100 ml capacity.

6.2 Apparatus for the hydrolysis of the triglycerides

6.2.1 Centrifuge (if necessary).

6.2.2 Centrifuge tube, 10 ml capacity, with ground stopper.

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

6.2.4 Water-bath, thermostatically controlled, and capable of being maintained at 40 ± 0,5 °C.

6.2.5 Hypodermic syringe, 1 ml capacity, with thin needle.

6.2.6 Stop-watch.

6.3 Apparatus for the isolation of the 2-monoglycerides

6.3.1 Developing tank, for thin layer chromatography, with ground glass lid, suitable for containing glass plates 200 mm × 200 mm.

6.3.2 Spreader and rack, for preparation of the plates.

6.3.3 Glass plates, 200 mm × 200 mm.

6.3.4 Microsyringe, capable of dispensing drops of 3 to 4 µl as a continuous uniform band.

1) Alternative names : tris-(hydroxymethyl) methylamine; tris-(hydroxymethyl) aminomethane.

6.3.5 Apparatus for spraying the indicator solution on to the plates.

6.3.6 Microspatula.

6.3.7 Oven, capable of being maintained at 103 ± 2 °C.

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

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

6.3.10 Conical flask, 250 ml capacity, with ground stopper.

6.3.11 Conical flask, 50 ml (if necessary).

6.3.12 Filter, sintered glass, porosity P 40 (16 to 40 µm) (if necessary).

6.3.13 Desiccator.

6.4 Apparatus for the analysis of the 2-monoglycerides by gas chromatography

See ISO 5508 and ISO 5509.

7 Sampling

See ISO 5555.

8 Procedure

8.1 Preparation of test sample

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

8.2 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 8.4.

If the acidity exceeds 3 % (m/m), first neutralize the sample with sodium hydroxide in the presence of solvent in accordance with 8.3, then purify through alumina in accordance with 8.4.

8.3 Neutralization with sodium hydroxide

Dissolve about 10 g of the test sample in 100 ml of hexane (if available) or light petroleum (5.1.2) and transfer the solution to the separating funnel (6.1.5). Add 50 ml of 2-propanol or ethanol 95 % (V/V) (5.1.1), a few drops of phenolphthalein solution (5.1.5) and a volume of sodium hydroxide solution (5.1.4) 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 neutralized oil with successive portions of 25 or 30 ml of a solution of 2-propanol or ethanol (5.1.3) until the pink colour of the phenolphthalein disappears.

Transfer the solution into the rotary evaporator flask (6.1.3) and remove most of the solvent by evaporating under reduced pressure. Dry the oil at 30 to 40 °C under reduced pressure using a nitrogen stream (5.1.7) until the solvent is completely removed.

8.4 Purification of the test portion through alumina

Prepare a suspension of 15 g of activated alumina (5.1.6) in 50 ml of hexane (if available) or light petroleum (5.1.2) and pour, while shaking, into a glass column for chromatography (6.1.2). Ensure that the alumina settles evenly and allow the solvent level to fall to 1 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 (5.1.2) and collect all the liquid which elutes from the column in a 100 ml round-bottom flask (6.1.6).

Remove most of the solvent by evaporation under reduced pressure, then dry the oil at 30 to 40 °C using a nitrogen stream (5.1.7) until the solvent is completely removed.

8.5 Hydrolysis of the triglycerides

Weigh approximately 0,1 g of the purified test portion (8.4) in a 10 ml centrifuge tube (6.2.2). If this test portion is solid, place the tube in a water-bath at 60—65 °C until it has liquefied or for not longer than 40 s, even if by then the test portion is not completely liquid. Bring the temperature of the tube to about 40 °C and continue the procedure without delay.

Place the centrifuge tube containing the purified test portion in the water-bath at 40 ± 0,5 °C. Add 2 ml of the buffer solution (5.2.5), 0,5 ml of sodium cholate solution (5.2.3) and 0,2 ml of calcium chloride solution (5.2.4) and shake carefully. Add 20 mg of lipase (5.2.6), insert the stopper and shake manually for exactly 1 min with the tube in the water-bath.

Remove the tube from the water-bath and shake vigorously for exactly 2 min using the shaker (6.2.3).

Add 1 ml of hydrochloric acid (5.2.2) and 1 ml of diethyl ether (5.2.1). Insert the stopper and shake vigorously using the shaker (6.2.3). Centrifuge and transfer the organic phase to a test-tube using the syringe (6.2.5). 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.

8.6 Isolation of the 2-monoglycerides

8.6.1 Preparation of the plates[1)]

Carefully clean the glass plates (6.3.3) with ethanol (5.3.1), hexane or light petroleum (5.3.2) and acetone (5.3.3) until any fatty matter is completely removed.

Weigh 30 g of silica (5.3.4) into a 250 ml conical flask (6.3.10). Add 60 ml of water. Insert the stopper and shake vigorously for 1 min. Immediately introduce the slurry into the spreader (6.3.2). Spread a layer 0,25 mm thick on the clean plates. Allow the plates to dry for at least 15 min in air.

In all cases, whether the plates have been prepared as above or commercially prepared, activate the plates at 103 ± 2 °C in an oven (6.3.7) for 1 h. Before use, allow the plates to cool to ambient temperature in a desiccator (6.3.13).

NOTE — 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 in a developing tank with the solvent (5.3.5) and allowing the solvent to reach the top of the plate.

8.6.2 Separation of the 2-monoglycerides

Using a microsyringe (6.3.4), transfer the extract (8.5) onto a prepared plate (8.6.1) as a continuous band of fine droplets 15 mm from one of the edges.

Stand the plate in the developing tank (6.3.1), which has previously been saturated with the developing solvent (5.3.5), 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 (5.3.6) using the apparatus (6.3.5). Mark the band of monoglycerides (R_f = 0,035 approximately) under ultraviolet light (6.3.8) and scrape off using a microspatula (6.3.6) (avoid removing the components which remain on the starting line).

For purified test portions (8.4) which were liquid at ambient temperature, transfer the collected silica to a 25 ml methylation flask (6.3.9) and proceed as described in 8.7.

For purified test portions (8.4) which were solid at ambient temperature, transfer the silica to a 50 ml conical flask (6.3.11) with 15 ml of diethyl ether. Shake vigorously and transfer all the silica to a sintered glass filter (6.3.12). 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 4 to 5 ml and transfer it to a previously weighed 25 ml round-bottom flask (6.3.9), then evaporate the solvent in a stream of nitrogen. Weigh the residue. The amount of monoglycerides obtained should be between 10 and 30 % (m/m) of the test portion; if this is not the case, repeat the hydrolysis of the triglycerides (8.5) or recheck the activity of the lipase (see the annex).

8.7 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 Test report

The test report shall show the method used and the results 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 the information necessary for the complete identification of the sample.

1) Fully prepared plates are commercially available.

Annex

Preparation of the lipase and checking its activity

(This annex does not form part of the standard.)

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 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 cholate solution (200 g/l) and 20 ml of distilled water successively into a 50 ml beaker.

Place the beaker in a water-bath maintained at 37 ± 0,5 °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 stop-watch 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 or 5 min. Such a result is usually obtained by using 1 to 5 ml of lipase suspension, i.e. 1 to 5 mg of powder.

The lipase unit is defined as the quantity of enzyme which is required to release 1 micro-equivalent 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

$$A = \frac{V \times 100}{m}$$

where

V is the volume, in millilitres, of 0,1 mol/l sodium hydroxide solution consumed per minute, calculated from the graph;

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.