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Animal and vegetable fats and oils — Preparation of methyl esters of fatty acids

Corps gras d'origines animale et végétale — Préparation des esters méthyliques d'acides gras

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FOREWORD

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It has been approved by the member bodies of the following countries :

Australia	Hungary	Poland
Austria	Iran	Romania
Canada	Israel	South Africa, Rep. of
Chile	Korea, Rep. of	Spain
Czechoslovakia	Mexico	Thailand
Ethiopia	Netherlands	Turkey
France	New Zealand	United Kingdom
Germany, F.R.	Peru	Yugoslavia

No member body expressed disapproval of the document.

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Animal and vegetable fats and oils — Preparation of methyl esters of fatty acids

1 SCOPE

This International Standard specifies methods of preparing the methyl esters of fatty acids.

The methyl esters so produced can be used in the various analytical procedures requiring such derivatives, for example gas-liquid chromatography, thin-layer chromatography, infra-red spectrophotometry, etc.

2 FIELD OF APPLICATION

2.1 The methods specified in clauses 4 and 5 are applicable to the preparation of methyl esters of fatty acids with 6 or more carbon atoms, from all types of animal and vegetable fats, oils and fatty acids. In the presence of fatty acids with 6 or 8 carbon atoms, and in the case of preparing methyl esters for gas-liquid chromatography, it is essential that the solvent should not be removed from the solution of methyl esters.

The general method using boron trifluoride (clause 4) is to be preferred for most oils and fats, but may lead to erroneous results in the following cases :

— compounds having secondary oxygen groupings (hydroxy-, hydroperoxy-, keto-, epoxy-);

— compounds containing cyclopropane- and cyclopropene- groups;

— conjugated polyunsaturated compounds and acetylenic compounds;

— waxes.

For these it is preferable to use one of the methods described in clause 5. Nevertheless, if the fatty matter contains such compounds only in very small amount (for example cottonseed oil), it may be esterified according to the general method in clause 4.

See also 8.1.

2.2 The special method described in clause 6 is applicable to the preparation of methyl esters of fatty acids with 4 or more carbon atoms, from neutral oils and fats (acid value less than 2), principally for analysis by gas-liquid chromatography.

3 REFERENCE

ISO/R 661, *Crude vegetable oils and fats — Preparation of contract sample for analysis.*

4 GENERAL METHOD USING BORON TRIFLUORIDE

WARNING — Boron trifluoride is poisonous. For this reason, it is not recommended that the analyst prepare the methanolic solution of boron trifluoride from methanol and boron trifluoride (see 8.3).

The methods described involve the use of potentially hazardous reagents. Normal precautions should be taken for eye protection and for protection from the dangers of corrosive chemical burns.

4.1 Principle

Saponification of the glycerides, and esterification of the liberated fatty acids in the presence of boron trifluoride.

4.2 Reagents

Unless stated otherwise, all reagents and solvents shall be of analytical quality, and the water shall be distilled water or water of equivalent quality.

4.2.1 Sodium hydroxide, methanolic solution, approximately 0,5 N.

Dissolve 2 g of sodium hydroxide in 100 ml of methanol containing not more than 0,5 % (*m/m*) of water. If the solution has to be stored for a considerable time, a small amount of white precipitate of sodium carbonate may be formed; this has no effect on the preparation of the methyl esters.

4.2.2 Boron trifluoride, methanolic solution, 12 % to 15 % (*m/m*)[1] (see 8.2).

4.2.3 Heptane, of chromatographic quality (see 8.2 and 8.4).

4.2.4 Light petroleum, redistilled (boiling range 40 to 60 °C), bromine value less than 1, residue-free, or **hexane** (see 8.2).

4.2.5 Sodium sulphate, anhydrous.

4.2.6 Sodium chloride, saturated aqueous solution.

4.2.7 Methyl red, 1 g/l solution in 60 % (*V/V*) ethanol.

4.2.8 Nitrogen, having an oxygen content less than 5 mg/kg.

4.3 Apparatus

Usual laboratory equipment, and in particular :

4.3.1 Flask, 50 ml or 100 ml, with ground neck.

4.3.2 Reflux condenser, 20 to 30 cm effective length, with ground joint to fit the flask (4.3.1).

4.3.3 Boiling aid, fat-free.

4.3.4 Graduated pipette, capacity at least 10 ml and fitted with a rubber bulb; or an **automatic pipette.**

4.3.5 Inlet tube for nitrogen.

4.3.6 Test tube with ground neck and fitted with a ground glass stopper.

4.3.7 Separating funnels, 250 ml.

4.4 Procedure

Because of the toxic character of boron trifluoride, the following operations are best performed under a ventilated hood. It is essential to wash all glassware with water immediately after use.

4.4.1 *Preparation of the test sample*

The test sample shall be dry and clear. Proceed therefore in accordance with ISO/R 661, but heat the sample to just above the melting point.

4.4.2 *Test portion*

Precise weighing is not normally necessary (see 8.8). The size of the test portion is only required in order that the appropriate size of flask (4.3.1) and the quantities of the reagents and solvent may be selected according to the following table :

Test portion	Flask (4.3.1)	NaOH solution (4.2.1)	BF_3 solution (4.2.2)	Heptane (4.2.3)
mg	ml	ml	ml	ml
100 to 250	50	4	5	1 to 3
250 to 500	50	6	7	2 to 5
500 to 750	100	8	9	4 to 8
750 to 1 000	100	10	12	7 to 10

If the methyl esters are intended for an analysis by gas-liquid chromatography, a test portion of about 350 mg is to be preferred (see 8.5). If it is smaller, care should be taken to ensure that the sample is representative.

4.4.3 *Saponification* (see 8.1)

4.4.3.1 General case of fats and oils

Introduce the test portion into the appropriate flask (see 4.4.2). Add the appropriate amount (see 4.4.2) of the methanolic sodium hydroxide solution (4.2.1) and a boiling aid (4.3.3). Fit the condenser (4.3.2) to the flask.

NOTE — In the presence of fatty acids containing more than two double bonds it is recommended that the air in the methanolic solution and in the flask be removed by bubbling nitrogen (4.2.8) through the solution for a few minutes and maintaining a current of nitrogen into the upper part of the condenser during the following saponification.

Boil under reflux until the droplets of fat disappear (this usually takes 5 to 10 min, but in certain exceptional cases it may take longer) (see 8.7). Add the appropriate amount (see 4.4.2) of the methanolic boron trifluoride solution (4.2.2) from the graduated pipette or automatic pipette (4.3.4) through the top of the condenser to the boiling liquid. Proceed in accordance with 4.4.4.

4.4.3.2 Special case of fatty acids

If the sample consists entirely of fatty acids, the saponification step is not necessary.

Introduce the test portion into the appropriate flask (see 4.4.2). Add the appropriate amount (see 4.4.2) of the methanolic boron trifluoride solution (4.2.2) from the

1) 14 and 50 % solutions are available commercially.

graduated pipette or automatic pipette (4.3.4). Fit the condenser (4.3.2) to the flask and bring to the boil.

4.4.4 *Preparation of the methyl esters*

4.4.4.1 Continue boiling for 2 min.

4.4.4.2 Add the appropriate amount (see 4.4.2) of the heptane (4.2.3) (see 8.4) to the boiling mixture through the top of the condenser (the precise amount does not affect the reaction), and continue boiling for 1 min.

Stop heating, cool to room temperature and then remove the condenser. Add a small portion of the saturated sodium chloride solution (4.2.6) and swirl the flask gently several times.

Add more saturated sodium chloride solution to the flask in order to bring the level of liquid into the neck of the flask.

4.4.4.3 Transfer about 1 ml of the upper layer (heptane solution) into a test tube (4.3.6) and add anhydrous sodium sulphate (4.2.5) to remove any traces of water.

This solution will contain about 100 mg/ml of methyl esters and may be injected directly into the column for gas-liquid chromatography (see 7.1).

4.4.4.4 If it is required that the whole of the dry esters should be recovered, transfer the saline solution and the heptane layer to a 250 ml separating funnel (4.3.7). Separate the layers. Retain the heptane solution. Extract the saline solution twice with 50 ml portions of light petroleum or hexane (4.2.4).

Combine the heptane solution and the two extracts, and wash them with 20 ml portions of water until free from acid, using the methyl red solution (4.2.7) as indicator. Dry over anhydrous sodium sulphate (4.2.5), filter and evaporate the solvent on a water bath under a stream of nitrogen (4.2.8) (see 8.6). For test portions less than 500 mg, it is preferable to reduce proportionately the volumes of solvent and water.

5 ALTERNATIVE METHODS NOT INVOLVING THE USE OF BORON TRIFLUORIDE

5.1 Method applicable to neutral fats and oils (acid value less than 2)

5.1.1 *Principle*

Methanolysis of the glycerides in an alkaline medium.

5.1.2 *Reagents*

Unless stated otherwise, all reagents and solvents shall be of analytical quality, and the water shall be distilled water or water of equivalent quality.

5.1.2.1 Methanol, containing not more than 0,5 % (*m/m*) of water.

5.1.2.2 Potassium hydroxide, methanolic solution, approximately 1 N.

Dissolve 5,6 g of potassium hydroxide in 100 ml of methanol (5.1.2.1).

5.1.2.3 Heptane, of chromatographic quality (see 8.2 and 8.4).

5.1.2.4 Sodium sulphate, anhydrous.

5.1.2.5 Nitrogen, having an oxygen content less than 5 mg/kg.

5.1.3 *Apparatus*

Usual laboratory equipment, and in particular :

5.1.3.1 High-speed stirrer and appropriate means of heating (for example a magnetic stirrer equipped with a heater).

5.1.3.2 Flask, 100 ml, with ground neck.

5.1.3.3 Inlet tube for nitrogen.

5.1.3.4 Reflux condenser, with ground joint to fit the flask (5.1.3.2).

5.1.3.5 Boiling aids, fat-free.

5.1.3.6 Separating funnels, 250 ml.

5.1.3.7 Conical flask, 50 ml, with narrow neck.

5.1.4 *Procedure*

5.1.4.1 PREPARATION OF THE TEST SAMPLE

The test sample shall be dry and clear. Proceed therefore in accordance with ISO/R 661, but heat the sample to just above the melting point.

5.1.4.2 TEST PORTION

Weigh approximately 4 g of the test sample (see 8.5).

5.1.4.3 PREPARATION OF THE METHYL ESTERS (see 8.1)

Introduce the test portion into the flask (5.1.3.2). Add about 40 ml of the methanol (5.1.2.1), 0,5 ml of the methanolic potassium hydroxide solution (5.1.2.2) and a boiling aid (5.1.3.5). Fit the condenser (5.1.3.4) to the flask.

NOTE – In the presence of fatty acids containing more than two double bonds, it is recommended that the air in the methanolic solution and in the flask be removed by bubbling nitrogen (5.1.2.5) through the solution for a few minutes and maintaining a current of nitrogen into the upper part of the condenser during the following saponification.

Bring to the boil. The solution should become clear. The reaction is normally complete after 5 to 10 min (see 8.7).

Cool the flask under running water, and transfer the contents to a separating funnel (5.1.3.6).

Rinse the flask into the separating funnel with 20 ml of the heptane (5.1.2.3) (see 8.4). Add about 40 ml of water, shake and allow to separate. The esters pass into the upper heptane layer. Draw off the aqueous layer into a second separating funnel and extract it again with 20 ml of heptane. Combine the two extracts and wash them twice with 20 ml portions of water. Separate and dry the ester solution over anhydrous sodium sulphate (5.1.2.4). Filter through cotton wool into a 50 ml conical flask (5.1.3.7) and evaporate the solution to approximately 20 ml over a boiling water bath, under a stream of nitrogen (see 8.6).

5.2 Method applicable to acid fats and oils (acid value greater than 2) and fatty acids

5.2.1 *Principle*

For acid fats and oils, neutralization of the free fatty acids, and alkaline methanolysis of the glycerides followed by esterification of the fatty acids in acid medium.

For fatty acids, esterification in an acid medium.

5.2.2 *Reagents*

Unless stated otherwise, all reagents and solvents shall be analytical quality, and the water shall be distilled water or water of equivalent quality.

5.2.2.1 Sodium methylate, methanolic solution.

Dissolve 1 g of sodium in 100 ml of methanol containing not more than 0,5 % (*m/m*) of water.

5.2.2.2 Hydrochloric acid, anhydrous methanolic solution, approximately 1 N.

NOTES

1 Small amounts of anhydrous gaseous hydrogen chloride can be prepared in the laboratory by displacement from the commercial solution (ρ_{20} 1,18 g/ml) by dripping the latter into concentrated sulphuric acid (ρ_{20} 1,84 g/ml). The liberated gas is dried by bubbling it through sulphuric acid. Since hydrochloric acid is rapidly absorbed by methanol, it is advisable to take the usual precautions in dissolving it; for example introduce the gas through a small inverted funnel with the rim just touching the surface of the liquid. Since methanolic hydrochloric acid solution keeps perfectly in glass-stoppered bottles stored in the dark, large quantities of the solution may be prepared in advance.

2 Approximately 1 N methanolic sulphuric acid solution may be used in place of the methanolic hydrochloric acid solution, but esterification then requires at least 20 min, and the precipitated sodium sulphate hinders boiling, so that either filtration or the use of a magnetic stirrer is necessary.

5.2.2.3 Heptane, of chromatographic quality (see 8.2 and 8.4).

5.2.2.4 Sodium sulphate, anhydrous.

5.2.2.5 Methyl red, 1 g/l solution in 60 % (*V/V*) ethanol.

5.2.2.6 Nitrogen, having an oxygen content less than 5 mg/kg.

5.2.3 *Apparatus*

Usual laboratory equipment, and in particular :

5.2.3.1 High-speed stirrer and appropriate means of heating (for example a magnetic stirrer equipped with a heater).

5.2.3.2 Flask, 250 ml, with ground neck.

5.2.3.3 Inlet tube for nitrogen.

5.2.3.4 Reflux condenser, with ground joint to fit the flask (5.2.3.2).

5.2.3.5 Boiling aid, fat-free.

5.2.3.6 Separating funnel, 250 ml.

5.2.3.7 Conical flask, 100 ml, with narrow neck.

5.2.4 *Procedure*

5.2.4.1 PREPARATION OF THE TEST SAMPLE

The test sample shall be dry and clear. Proceed therefore in accordance with ISO/R 661, but heat the sample to just above the melting point.

5.2.4.2 TEST PORTION

Weigh approximately 4 g of the test sample (see 8.5).

5.2.4.3 METHANOLYSIS (see 8.1)

5.2.4.3.1 Case of acidic fats and oils

Introduce the test portion into the flask (5.2.3.2). Add 40 ml of the sodium methylate solution (5.2.2.1) and a boiling aid (5.2.3.5). Fit the condenser (5.2.3.4) to the flask.

NOTES

1 Alternatively, place 40 ml of methanol and 0,4 g of sodium in the flask before introducing the test portion, thus preparing *in situ* a solution of sodium methylate.

2 In the presence of fatty acids containing more than two double bonds, it is recommended that the air in the methanolic solution and in the flask be removed by bubbling nitrogen (5.2.2.6) through the solution for a few minutes and maintaining a current of nitrogen into the upper part of the condenser during the following saponification.

Bring to the boil. The solution should become clear. The reaction is normally complete in less than 15 min (see 8.7).

Add at least 50 ml of the methanolic hydrochloric acid solution (5.2.2.2) to the flask and proceed in accordance with 5.2.4.4.

NOTE – Owing to the relatively high quantity of sodium methylate, precipitation of sodium chloride occurs, which may lead to superheating ("bumping") during the subsequent boiling. This precipitate may be filtered off, although, owing to the short period of boiling prescribed, this is usually unnecessary.

5.2.4.3.2 Case of fatty acids

If the sample consists entirely of fatty acids, the saponification step is not necessary.

Introduce the test portion into the flask (5.2.3.2). Add 50 ml of the methanolic hydrochloric acid solution (5.2.2.2), and a boiling aid (5.2.3.5). Fit the condenser (5.2.3.4) to the flask.

NOTE – In the presence of fatty acids containing more than two double bonds, it is recommended that the air in the methanolic solution and in the flask be removed by bubbling nitrogen (5.2.2.6) through the solution for a few minutes and maintaining a current of nitrogen into the upper part of the condenser during the following boiling.

5.2.4.4 PREPARATION OF THE METHYL ESTERS

Boil for 10 min, then cool the flask under running water, add 100 ml of water to the flask, then transfer the contents to the 250 ml separating funnel (5.2.3.6) and add 30 ml of the heptane (5.2.2.3) (see 8.4). Shake vigorously and allow to settle until the two phases have separated. Collect the heptane layer. Extract the aqueous phase again with 30 ml of heptane. Combine the two heptane extracts and wash them with 20 ml portions of water until free from acid, using the methyl red solution (5.2.2.5) as indicator. Dry over anhydrous sodium sulphate (5.2.2.4). Filter through cotton wool into the 100 ml conical flask (5.2.3.7) and evaporate the solution to approximately 20 ml over a boiling water bath, under a stream of nitrogen (see 8.6).

6 SPECIAL METHOD FOR PREPARATION OF METHYL ESTERS OF FATTY ACIDS WITH 4 OR MORE CARBON ATOMS

6.1 Principle

Transesterification of the glycerides by reaction with a methanolic potassium hydroxide solution.

6.2 Reagents

Unless stated otherwise, all reagents and solvents shall be of analytical quality, and water shall be distilled water or water of equivalent quality.

6.2.1 Potassium hydroxide, methanolic solution, approximately 2 N.

Dissolve 11,2 g of potassium hydroxide in 100 ml of methanol containing not more than 0,5 % (*m/m*) of water.

6.2.2 Heptane, of chromatographic quality (see 8.2 and 8.4).

6.2.3 Reference solution I

Weigh, to the nearest 0,1 mg, about 1 g of methyl pentanoate into a 50 ml volumetric flask.

Dilute to the mark with heptane (6.2.2).

6.2.4 Reference solution II

Weigh, to the nearest 0,1 mg, about 200 mg of methyl pentanoate into a 100 ml volumetric flask.

Dilute to the mark with heptane (6.2.2).

6.3 Apparatus

Usual laboratory equipment, and in particular :

6.3.1 Test tube, 20 ml, with ground glass stopper.

6.3.2 Volumetric flasks, 50 ml and 100 ml.

6.3.3 Graduated pipette, capacity 1 ml or more.

6.3.4 Measuring cylinder, 10 ml.

6.4 Procedure

6.4.1 *Preparation of the test sample*

The test sample shall be dry and clear. Proceed therefore in accordance with ISO/R 661, but heat the sample to just above the melting point.

6.4.2 *Test portion*

Weigh, to the nearest 1 mg, 1 g of the test sample, in the test tube (6.3.1).

NOTE – If it is not intended to determine the butyric acid content, it is not necessary to weigh the test portion to the specified accuracy.

6.4.3 *Preparation of the methyl esters*

6.4.3.1 Add to the test tube 10 ml of heptane (6.2.2), from the measuring cylinder (6.3.4).

6.4.3.2 If it is intended to carry out a subsequent accurate determination of the butyric acid content by gas-liquid chromatography, and only in this case, add to the test tube, using the graduated pipette (6.3.3), 1,0 ml of reference solution I (6.2.3) if the expected butyric acid content is above 1 % (*m/m*), or 1,0 ml of reference solution II (6.2.4) if the expected butyric acid content is less than 1 % (*m/m*).

6.4.3.3 Add 0,5 ml of the methanolic potassium hydroxide solution (6.2.1), stopper the test tube and mix the contents by shaking until the solution becomes clear; this will take about 20 s. Just after this clarification, the solution becomes cloudy again as a result of the separation of glycerol. Settling of the glycerol takes place rapidly.

Decant the upper layer, containing the methyl esters.

7 STORAGE OF METHYL ESTERS

7.1 The esters should preferably be analysed as soon as possible.

If necessary, the heptane solution containing the methyl esters may be stored under inert gas and in a refrigerator. For a longer period of storage, it is advisable to protect the methyl esters against autoxidation by adding to the solution an antioxidant in such concentration as will not interfere with the subsequent analysis, for example a 0,05 g/l solution of BHT (2,6-*di-tert* butyl-4-methyl phenol).

Methyl esters containing methyl butyrate can only be stored in sealed ampoules, and it is essential to take very special precautions to prevent any loss by evaporation during filling and sealing of the ampoules.

7.2 The dry methyl esters without solvent should be analysed without delay. If required, they may be kept for 24 h under inert gas in a refrigerator, or longer under vacuum in a sealed tube in a freezer.

8 REMARKS ON REAGENTS AND PROCEDURES

8.1 Unsaponifiable matter is not removed and, if it is present in substantial amount, it may interfere with subsequent analysis. If this is the case, it is essential to supplement the method described with the following operations :

Dilute with water the solution obtained after saponification and extract the unsaponifiable matter with diethyl ether, hexane or light petroleum. Acidify the solution and separate the fatty acids. Prepare the methyl esters from these as described in 4.4.3.2 or 5.2.4.3.2.

8.2 The various reagents and solvents must not produce peaks which interfere with those of the methyl esters of fatty acids during gas-liquid chromatography. In the course of the gas-liquid chromatography of the methyl esters, certain reagents, particularly the methanolic boron trifluoride solution, may produce adventitious peaks on the graph (in the region of the $C_{20} - C_{22}$ acids in the case of methanolic boron trifluoride solutions). Consequently, any new batch of reagent or solvent should be checked by preparing the methyl esters of pure oleic acid, and chromatographing them; if an extraneous peak appears, the reagent should be rejected.

8.3 If it is absolutely essential to prepare a boron trifluoride methanolic solution, proceed as follows. Weigh a 2 litre flask containing 1 litre of methanol. Cool in a bath of iced water. Keeping the flask in the bath, bubble BF_3 from a gas cylinder through a glass tube into the methanol until 125 g have been absorbed, operating under a hood. Pass the stream of BF_3 through the glass tube before immersing the latter in the methanol and until it is removed, in order to prevent any liquid from returning to the gas expansion system. The gas should not give rise to white fumes by escaping too quickly from the flask. The reagent remains stable for 2 years if stored in a refrigerator.

8.4 If fatty acids containing 20 or more carbon atoms are absent, hexane may be substituted for heptane.

8.5 If the suggested amount of sample is not available, smaller amounts down to 10 mg, or even less, may be used, provided that the amounts of reagent and the size of the apparatus are reduced proportionately.

8.6 There is some risk of losing part of the most volatile esters if the evaporation of the solvent is prolonged, or if the current of nitrogen is too vigorous.

For infra-red spectrophotometry, it is essential that elimination of the solvent be as complete as possible. For gas-liquid chromatography, and if fatty acids with 8 or fewer carbon atoms are present, the solvent must not be removed.

8.7 In the case of oils (such as castor oil) which are soluble in methanol, no droplets of oil will be observed, and consequently clarity is not the criterion for judging completion of the reaction.

8.8 If fatty acids are to be determined quantitatively by gas-liquid chromatography using internal standard(s), it is essential to weigh the test portion accurately (i.e. to the nearest 1 mg).