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Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters —

Part 2:
Preparation of methyl esters of fatty acids

Corps gras d'origines animale et végétale — Chromatographie en phase gazeuse des esters méthyliques d'acides gras —

Partie 2: Préparation des esters méthyliques d'acides gras

Reference number
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Case postale 56 • CH-1211 Geneva 20
Tel. + 41 22 749 01 11
Fax + 41 22 749 09 47
E-mail copyright@iso.org
Web www.iso.org

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Foreword

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International Standards are drafted in accordance with the rules given in the ISO/IEC Directives, Part 2.

The main task of technical committees is to prepare International Standards. 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.

Attention is drawn to the possibility that some of the elements of this document may be the subject of patent rights. ISO shall not be held responsible for identifying any or all such patent rights.

ISO 12966-2 was prepared by Technical Committee ISO/TC 34, *Food products*, Subcommittee SC 11, *Animal and vegetable fats and oils*.

This first edition of ISO 12966-2 cancels and replaces ISO 5509:2000, of which it constitutes a technical revision.

ISO 12966 consists of the following parts, under the general title *Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters*:

- *Part 2: Preparation of methyl esters of fatty acids*
- *Part 3: Preparation of methyl esters using trimethylsulfonium hydroxide (TMSH)*

The following part is under preparation:

- *Part 4: Determination of cis-, trans-, saturated, mono- and polyunsaturated fatty acids in vegetable or non-ruminant oils and fats*

The following part is planned:

- *Part 1: Guidelines on gas chromatography*

Introduction

General

Oils and fats (i.e. liquid and solid lipids) are predominantly composed of fatty acid esters of glycerol (triacylglycerols, TAGs), with smaller amounts of fatty acid esters of sterols and long chain aliphatic alcohols. Due to the high molecular mass of the TAGs and their consequent low volatility, they are difficult to analyse directly by gas chromatography (GC), especially if a detailed analysis of unsaturated fatty acids is required. Fatty acids themselves do not chromatograph well (except for short-chain-length fatty acids, e.g. butanoic and pentanoic acids). It is therefore better practice to form fatty acid esters, usually the fatty acid methyl esters (FAMEs), prior to GC.

The analysis of oils and fats has been extensively reviewed in Reference [9].

The formation of FAMEs is a critical stage in the analysis of fatty acids. Non-quantitative conversion of fatty acids to FAMEs, modification of the structure of fatty acids (e.g. changes in positional and geometric isomers present) and formation of non-FAME artefacts may all affect the quantitative determination of fatty acid composition.

Transesterification is one mechanism which can be employed to form FAMEs from fatty acid esters in fats (i.e. triacylglycerol). Alkali- or acid-catalysed transesterification procedures can be used to form FAMEs in a methanolic medium; the procedure can be termed *transmethylation*. Transmethylation is a reversible process and a large excess of methanol is required to maintain an equilibrium position which favours formation of the FAMEs. Water can prevent the reaction going to completion, and its presence should therefore be minimized. Alkali-catalysed procedures do not produce FAMEs from free fatty acids, due to the formation of soaps.

Esterification is an acid-catalysed mechanism which can be employed to form FAMEs from fatty acids. It is possible that the fatty acids are naturally present in the sample of fat under examination. Formation of FAMEs by this mechanism is commonly termed *methylation*. Again, an excess of methanol and the absence of water are preconditions for the quantitative formation of FAMEs.

This part of ISO 12966 provides guidelines for the preparation of fatty acid methyl esters. In support of these guidelines, various procedures to prepare fatty acid methyl esters are specified. These include:

a) “rapid” transmethylation under alkaline conditions;

b) “general” transmethylation/methylation under sequential alkaline and acid conditions;

c) boron trifluoride (BF_3) transmethylation/methylation.

“Rapid” transmethylation method under alkali-catalysed conditions

This method is applicable to the routine analysis of edible fats and oils containing fatty acids down to butanoic acid (C4:0) and/or for the determination of butanoic acid or hexanoic acid (C6:0) by GC using an internal standard.

Alkaline catalysts transesterify neutral lipids in the presence of anhydrous methanol (transmethylation) more rapidly than acid catalysts. The disadvantages of such alkali-catalysed procedures are that free fatty acids are not esterified, and the presence of water may prevent the transmethylation going to completion (hydrolysis of the FAMEs to free fatty acids). The most commonly used reagents are potassium and sodium hydroxide and sodium methoxide in the presence of anhydrous methanol.

“General” transmethylation/methylation under sequential alkaline and acid conditions

This method under sequential alkali- and acid-catalysed conditions is applicable to all oils and fats including distillate and acid oils, but is not recommended for lauric oils. Short-chain fatty acid methyl esters are easily lost during reflux. For lauric acid oils, the “rapid” transmethylation method is recommended.

During methylation, substances containing the following configurations can be totally or partially decomposed:

a) keto, epoxy, hydroxyl, hydroperoxy groups;

b) cyclopropyl and cyclopropenyl groups;

c) acetylenic fatty acids.

Boron trifluoride (BF_3) transmethylation/methylation

Owing to the toxicity of BF_3 it is recommended that this method only be used *in extremis*.

The BF_3 method is applicable for most oils, fats and derivatives (fatty acids, soaps) with the exception of milk fats and fats containing fatty acids with specific groups.

During methylation, substances containing the following configurations can be totally or partially decomposed:

a) keto, epoxy, hydroxyl, hydroperoxy groups;

b) cyclopropyl and cyclopropenyl groups;

c) acetylenic fatty acids.

If the fatty matter contains such substances in only very small amounts (e.g. cottonseed oil), the method can be applied, otherwise the “rapid” or “general” transmethylation/methylation methods should be followed.

For GC, the optimum recovery of the methyl esters from the reaction mixture is obtained by using isooctane (2,2,4-trimethylpentane). However, only about 75 % of the methyl caproate present is recovered.

Boron trifluoride is a strong Lewis acid, and in the form of its coordination complex with methanol, under reflux conditions, it can rapidly methylate fatty acids. Methanolic boron trifluoride does transmethylate fatty acid esters (e.g. triglyceride), but the rate of reaction is slower than the methylation of fatty acids. Methanolic boron trifluoride solution is commercially available, which enhances the attractiveness of this acid catalyst, but there are potential disadvantages associated with the use of this reagent.

a) It has been reported that high concentrations of boron trifluoride (50 % mass fraction) produce methoxy artefacts from unsaturated fatty acids.

b) The reagent has a limited shelf-life at ambient temperature and should be kept refrigerated.

c) Aged reagent may produce artefacts and therefore it is recommended that each new batch purchased be tested before use and periodically during its lifetime.

d) Methanolic boron trifluoride is an acidic reagent and therefore may produce derivatives of fatty acids containing labile groups which may give rise to spurious peaks on FAME chromatograms.

Additional information

Much attention has been given to the preparation and analysis of esters of short-chain fatty acids by GC, largely because of their occurrence in milk fats. Short-chain fatty acids, in the free state or esterified to glycerol, can be converted completely to methyl esters by any of the reagents described in the preceding paragraphs, but quantitative recovery from the reaction medium may not be achieved unless special precautions are taken. Losses can occur at several stages in any procedure. Short-chain fatty acid esters (methyl especially) are volatile and may be lost selectively on refluxing the esterification medium, they are more soluble in water than longer-chain esters and can be lost in an aqueous extraction step or they may be distilled off when the extracting solvent is evaporated. Selective losses can also occur if non-saponifiable impurities have to be removed by sublimation or thin-layer chromatography (TLC) purification. The best esterification procedures for short-chain fatty acids are those in which heating of the reagents is avoided and in which stages involving aqueous extraction and solvent removal are absent.

Injection of reaction media containing basic and acidic esterification catalysts directly on to GC columns shortens their working lives. The top few centimetres of packed columns can be replenished periodically, while lengths of deactivated tubing or “retention gaps” ahead of capillary columns protect them. This can be a small price to pay for the speed, simplicity, and accuracy of these procedures.

Additionally, this part of ISO 12966 gives a simple TLC procedure to check the effectiveness of the transmethylation/methylation. This procedure may also be used to check the generic composition of an oil or fat before transmethylation/methylation is undertaken.

Animal and vegetable fats and oils — Gas chromatography of fatty acid methyl esters —

Part 2:
Preparation of methyl esters of fatty acids

1 Scope

This part of ISO 12966 specifies methods of preparing the methyl esters of fatty acids.

It includes methods for preparing fatty acid methyl esters from animal and vegetable fats and oils, fatty acids and soaps. To cover different requirements four methylation methods are specified, namely:

a) a “rapid” transmethylation procedure under alkaline conditions;

b) a “general” transmethylation/methylation procedure under sequential alkaline and acid conditions;

c) a BF_3 transmethylation procedure;

d) an alternative procedure using acid-catalysed transmethylation of glycerides.

Methyl esters so produced are used in various analytical procedures requiring such derivatives, e.g. gas-liquid chromatography (GLC), thin-layer chromatography (TLC), and infrared spectrometry (IR).

2 Normative references

The following referenced documents are indispensable for the application of this document. 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*

3 Reactions

The determination of the fatty acid composition of oils and fats is one of the fundamental analyses within the fats and oils sector and has been extensively reviewed in Reference [9]. For this purpose, the fatty acid components of lipids are usually converted to methyl esters followed by GC analysis.

The “rapid” method (4.2) does not derivatize free fatty acids (FFAs) present in oil to fatty acid methyl esters (FAMEs). If FFAs are present, the assumption is usually made that the FFAs have the same fatty acid distribution as the triglycerides. This is usually true for crude oils, but less so for fractionated or refined oils. Except for some cold-pressed oils, as a general rule oils with <0,5 % mass fraction FFAs have probably been refined; oils above can be assumed to be crude. The tolerable concentration of FFAs in oil depends on the particular oil being analysed and also the intended use of the FAME data generated. The presence of FFAs in oil might introduce additional peaks on the final gas chromatogram and make the identification of FAMEs synthesized using the “rapid” transmethylation procedure problematic.

The “general” (4.3) procedure derivatizes both FFAs and glyceryl esters to FAMEs (see 4.3.1).

It is up to the analyst to decide whether the use of either the “rapid” or “general” procedure is appropriate based on the nature of the oil being analysed. This notwithstanding, as a general rule, use of the “rapid” method is suggested only if the FFA content is $\leqslant$0,5 % mass fraction. The “general” method (4.3) is suggested for oils with an FFA content >0,5 % mass fraction. Alternatively, if a partially hydrolysed fat is to be converted to FAMEs, the acid-catalysed transmethylation procedure specified in 4.5 may be used.

Owing to the toxicity of BF_3, it is recommended that the BF_3 method (4.4) be used only *in extremis*.

4 Methodology

WARNING — The method specified involves the use of potentially hazardous reagents. Normal precautions shall be taken for eye protection and for protection from the dangers of corrosive chemical burns. Methanolic potassium hydroxide solution is poisonous.

4.1 Preparation of test sample

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

4.2 Rapid method

4.2.1 Applicability

This rapid transmethylation method, under alkali-catalysed conditions, is applicable to the routine analysis of edible fats and oils containing fatty acids down to butanoic acid (C4:0) and/or for the determination of butanoic acid or hexanoic acid (C6:0) by GC using an internal standard.

NOTE 1 This procedure does not derivatize FFAs to FAMEs. The analyst should note that the presence of FFAs in the final solution can affect the quality of subsequent gas chromatography.

NOTE 2 According to COI/T.20/Doc. No. 24:2001[8], a similar procedure can be applied directly to samples of the following oil categories:

a) virgin olive oil with an acidity less than 3,3 %;

b) refined olive oil;

c) olive oil (blend of virgin and refined olive oil);

d) refined olive-pomace oil;

e) olive-pomace oil (blend of virgin olive oil and refined olive-pomace oil).

4.2.2 Principle

Methyl esters are formed by transmethylation with methanolic potassium hydroxide. Free fatty acids are not esterified by this procedure.

4.2.3 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified.

The reagents shall not produce peaks which interfere with those of the fatty acid methyl esters during GC. Any new batch of reagent or solvent should be checked by using it to prepare the methyl ester of pure oleic acid. If any extra, unexpected, peaks appear during the final GC analysis the reagent should be rejected.

4.2.3.1 **Methanol**, containing not more than 0,5 % mass fraction water.

4.2.3.2 **Water**, complying with ISO 3696[3], grade 3.

4.2.3.3 **Sodium hydrogensulfate**, anhydrous.

4.2.3.4 **Isooctane (2,2,4-trimethylpentane)**, chromatographic quality.

WARNING — Isooctane is flammable and a fire risk. Explosive volume fraction limits in air are 1,1 % to 6,0 %. It is toxic by ingestion and inhalation. Use a properly operating ventilated hood when working with this solvent.

4.2.3.5 **Potassium hydroxide**, methanolic solution, amount of substance concentration $c \approx 2$ mol/l.

Dissolve, with gentle heating, 13,1 g of potassium hydroxide (mass fraction $w = 85$ g/100 g) in 100 ml of absolute methanol.

4.2.3.6 **Internal standard stock solution**, for butanoic and/or hexanoic acid determination only.

Weigh 250 mg (to the nearest 0,1 mg) of valeric acid methyl ester (methyl pentanoate) into a 50 ml one-mark volumetric flask (4.2.4.4). Use isooctane to dissolve the sample and make up to the mark with the same solvent.

4.2.3.7 **Internal standard reference solution**, for butanoic and/or hexanoic acid determination only.

Add (4.2.4.2) 10 ml of stock solution to a 100 ml volumetric flask (4.2.4.4) and make up to the mark with isooctane. Calculate the concentration of this reference solution.

4.2.3.8 **Sodium chloride solution**. Dissolve 40 g of sodium chloride in 100 ml of water.

4.2.4 Apparatus

Usual laboratory apparatus and, in particular, the following.

4.2.4.1 **Screw-top test tubes**, 10 ml, with cap fitted with a PTFE-joint.

4.2.4.2 **Pipettes**, capacities 0,1 ml, 2 ml and 10 ml, ISO 8655-2[6].

4.2.4.3 **Glass sample vials**, 3 ml.

4.2.4.4 **One-mark volumetric flasks**, capacities 50 ml and 100 ml, ISO 1042[2] class A.

4.2.5 Procedure

In a 10 ml screw-top test tube (4.2.4.1), weigh approximately 0,1 g of the test sample (4.1). If fatty acids are to be determined quantitatively by GC using internal standard(s), it is essential to weigh the test portion accurately; i.e. to the nearest 0,1 mg. The results are then expressed as percentage mass fractions of the fatty acid in the fat or oil. These results do not necessarily agree with results obtained by internal normalization.

Add (4.2.4.2) 2 ml of isooctane (4.2.3.4), and shake. In certain instances, it may not be possible to use the specified 0,1 g sample size, in which case the amount of isooctane added to the test portion should be changed proportionately.

For butanoic and/or hexanoic acid determination, pipette (4.2.4.2) 2 ml of reference solution (4.2.3.7) instead of isooctane. In certain instances, it may be necessary to analyse fats and oils that contain low amounts of these fatty acids. In this instance, the volume of reference solution added to the test sample may be reduced proportionately.

Add (4.2.4.2) 0,1 ml of 2 mol/l methanolic potassium hydroxide solution (4.2.3.5), immediately put on the cap fitted with a PTFE-joint, tighten the cap, and shake vigorously for 1 min. The solution becomes clear and then shortly afterwards becomes cloudy again as glycerol separates. Allow to stand for approximately 2 min. Add approximately 2 ml of sodium chloride solution and shake briefly. Draw off the isooctane layer and transfer to a sample vial (4.2.4.3). Add approximately 1 g of sodium hydrogensulfate (4.2.3.3) and shake the solution.

The isooctane solution is suitable for analysis using GC in accordance with ISO 12966-4.

NOTE The effectiveness of derivatization using the "rapid" procedure can be determined by TLC as described in Annex A.

4.3 General method

4.3.1 Applicability

This general transmethylation/methylation method, under sequential alkali- and acid-catalysed conditions, is applicable to all oils and fats including distillate and acid oils, but not recommended for lauric oils. Short-chain fatty acid methyl esters are easily lost during reflux. For lauric oils, the method specified in 4.2 is recommended.

During methylation, substances containing the following configurations can be totally or partially decomposed:

a) keto, epoxy, hydroxyl, hydroperoxy groups;

b) cyclopropyl and cyclopropenyl groups;

c) acetylenic fatty acids.

NOTE According to COI/T.20/Doc. No. 24:2001[8], this method can be applied directly to samples of the following oil categories:

a) virgin olive oil with an acidity greater than 3,3 %;

b) crude olive-pomace oil.

4.3.2 Principle

The alkaline reagent causes transmethylation of glyceryl esters to fatty acid methyl esters; free fatty acids are converted to soaps. The acid catalyst converts the soaps to fatty acid methyl esters.

CAUTION — Methylation shall be done under a ventilated hood.

4.3.3 Reagents

Use only reagents of recognized analytical grade. The reagents shall not produce peaks which interfere with those of the fatty acid methyl esters during GC. Any new batch of reagent or solvent should be checked by using it to prepare the methyl esters of pure oleic acid. If any extra, unexpected, peaks appear during the final GC analysis the reagent should be rejected.

4.3.3.1 Isooctane (2,2,4-trimethylpentane), chromatographic quality.

WARNING — Isooctane is flammable and a fire risk. Explosive volume fraction limits in air are 1,1 % to 6,0 %. It is toxic by ingestion and inhalation. Use a properly operating ventilated hood when working with this solvent.

4.3.3.2 Methanol, containing not more than 0,05 % mass fraction water.

4.3.3.3 Sodium methoxide, methanolic solution, 0,2 mol/l.

Dissolve 8 g of sodium hydroxide in 1 000 ml of methanol. This may be prepared from commercial solutions.

4.3.3.4 Phenolphthalein, methanolic solution, 0,2 % mass fraction.

4.3.3.5 Sulfuric acid, methanolic solution, 1 mol/l.

Add 3 ml of 96 % sulfuric acid to 100 ml of methanol.

4.3.3.6 Sodium chloride solution.

Dissolve 40 g of sodium chloride in 100 ml of water.

4.3.3.7 Water, complying with ISO 3696[3], grade 3.

4.3.4 Apparatus and materials

Usual laboratory equipment, and in particular, the following.

4.3.4.1 One-mark volumetric flasks, capacity 10 ml with long, narrow, ground neck with a ground-glass stopper, ISO 1042[2] class A.

4.3.4.2 Reflux condenser, ISO 4799[4], with ground-glass joint to fit the neck of the flask.

4.3.4.3 Boiling chips, fat free.

4.3.4.4 Glass funnel.

4.3.4.5 Pipettes, capacities 0,2 ml, 1 ml, and 4 ml, ISO 8655-2[6].

4.3.5 Procedure

Transfer about 50 mg of the test sample (4.1) into a 10 ml ground-glass necked one-mark volumetric flask (4.3.4.1).

With the aid of a funnel (4.3.4.4), add 2 ml of 0,2 mol/l sodium methoxide in methanol (4.3.3.3) and the boiling chips (4.3.4.3).

Fit a reflux condenser (4.3.4.2), shake, and bring to the boil. Reflux the mixture until the solution becomes clear. For most oils this takes about 5 min, but for harder or long-chain saturated oils, it can take as long as 20 min.

NOTE 1 Sterol esters are also saponified.

Remove the flask from the source of heat, wait until the reflux stops, remove the condenser, and add two drops of phenolphthalein solution (4.3.3.4). Add sufficient 1 mol/l sulfuric acid in methanol solution (4.3.3.5) until the solution becomes colourless and then add (4.3.4.5) 0,2 ml in excess.

Fit the condenser and boil again for 5 min. Withdraw from the source of heat and cool the flask under running water. Remove the condenser, add (4.3.4.5) 4 ml of sodium chloride solution (4.3.3.6), and shake.

NOTE 2 Long reflux times under acidic conditions can exacerbate losses of dodecanoic acid.

Add (4.3.4.5) 1 ml of isooctane, plug the flask, and shake vigorously for 15 s. Leave to settle until the two phases have separated. Add sodium chloride solution again until the aqueous layer reaches the lower end of the flask neck. The upper layer containing the methyl esters fills the flask neck.

The upper isooctane layer is suitable for analysis using GC according to ISO 12966-4.

NOTE 3 The effectiveness of derivatization using the “general” procedure can be determined by TLC as described in Annex A.

4.4 Transmethylation using boron trifluoride (BF_3) catalyst

WARNING — The method described involves the use of potentially hazardous reagents. Normal precautions shall be taken for eye protection and for protection from the dangers of corrosive chemical burns.

IMPORTANT — 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.

4.4.1 Principle

In the first, alkali-catalysed, step, the TAGs are transmethylated with methanolic sodium hydroxide to form FAMEs. Any FFAs present are converted to soaps. In the second, acid-catalysed, step, the soaps are converted into methyl esters by reaction with a boron trifluoride-methanol complex.

Consequently, for analysis of pure fatty acids and soaps, the first, alkali-catalysed, step is unnecessary and FAMEs can be prepared directly by reaction with boron trifluoride.

4.4.2 Applicability

This method is applicable for most oils, fats and derivatives (fatty acids, soaps) with the exception of milk fats and of fats containing fatty acids with specific groups.

During methylation, substances containing the following configurations can be totally or partially decomposed:

a) keto, epoxy, hydroxyl, hydroperoxy groups;

b) cyclopropyl and cyclopropenyl groups;

c) acetylenic fatty acids.

If the fatty matter contains such substances in only very small amounts (e.g. cottonseed oil), the method can be applied, otherwise the methods specified in 4.2 or 4.3 should be followed.

For GC, the optimum recovery of the methyl esters from the reaction mixture is obtained by using isooctane. However, only about 75 % of the methyl caproate present is recovered.

4.4.3 Reagents

Use only reagents of recognized analytical grade.

The reagents shall not produce peaks which interfere with those of the fatty acid methyl esters during GC. Any new batch of reagent or solvent should be checked by using it to prepare the methyl esters of pure oleic acid. If any extra, unexpected, peaks appear during the final GC analysis the reagent should be rejected.

4.4.3.1 **Water**, complying with ISO 3696[3], grade 3.

4.4.3.2 **Sodium hydroxide**, methanolic solution, approximately 0,5 mol/l.

Dissolve 2 g of sodium hydroxide in 100 ml of methanol containing not more than 0,5 % mass fraction of water.

NOTE If the solution has to be stored for a considerable time, a small amount of white precipitate of sodium carbonate can be formed; this has no effect on the preparation of the methyl esters.

4.4.3.3 **Boron trifluoride (BF_3)**, methanolic solution, 12 % mass fraction to 15 % mass fraction.

4.4.3.4 **Isooctane (2,2,4-trimethylpentane)**, of chromatographic quality.

WARNING — Isooctane is flammable and a fire risk. Explosive limits in air are 1,1 % to 6,0 % (volume fraction). It is toxic by ingestion and inhalation. Use a properly operating ventilated hood when working with this solvent.

4.4.3.5 **Sodium chloride**, saturated aqueous solution.

4.4.3.6 **Sodium sulfate**, anhydrous.

4.4.3.7 **Nitrogen**, having an oxygen content <5 mg/kg.

4.4.3.8 **Hexane**, of chromatographic quality, for dry methyl esters only.

NOTE Light petroleum, boiling range 40 °C to 60 °C, redistilled and residue-free, with a bromine value less than 1, can be used.

4.4.3.9 **Methyl red**, 1 g/l solution in 60 % volume fraction ethanol.

NOTE Solutions of 14 %, 20 % and 50 % are available commercially.

4.4.4 Apparatus

Usual laboratory equipment and, in particular, the following.

4.4.4.1 **One-mark volumetric flasks**, capacity 50 ml or 100 ml, with ground neck and fitted with a ground-glass stopper, ISO 1042[2] class A.

4.4.4.2 **Reflux condenser**, ISO 4799[4], effective length 20 cm to 30 cm, with a ground-glass joint to fit the flask (4.4.4.1).

4.4.4.3 **Boiling chips**, fat-free.

4.4.4.4 **Graduated pipettes**, capacity 10 ml, ISO 835[1], fitted with a rubber bulb, or **automatic pipettes**, capacity 10 ml, ISO 8655-2[6] and fitted with a rubber bulb.

4.4.4.5 **Vials**, 4 ml, with screw cap.

4.4.4.6 **Separating funnels**, capacity 250 ml, ISO 4800[5], for dry methyl esters only.

4.4.4.7 **Rotary evaporator**.

4.4.4.8 **Analytical balance**, capable of being read to the nearest 0,001 g.

4.4.5 Procedure

WARNING — Because of the toxic character of boron trifluoride, perform the methylation under a ventilated hood. It is essential to wash all glassware with water immediately after use.

4.4.5.1 Test portion

Use Table 1 to select the appropriate size of flask and volumes of reagents and solvent required to methylate the mass of test portion to be taken from the test sample (4.1).

4.4.5.2 Saponification

For fats and oils, introduce the test portion chosen from Table 1 into the appropriate flask (4.4.4.1). Add the appropriate amount (see Table 1) of the methanolic sodium hydroxide solution (4.4.3.2) and a boiling chip. Fit the condenser (4.4.4.2) to the flask. Remove the air from the flask by flushing the flask with dry nitrogen (4.4.3.7) immediately prior to the reflux for a few minutes. Boil under reflux until the droplets of fat disappear, swirling the flask gently every 30 s to 1 min to prevent a solid ring of sodium hydroxide forming around the walls of the flask. This usually takes 5 min to 10 min, but in certain exceptional cases it can take longer.

NOTE In oils which are soluble in methanol, such as castor oils, no droplets of oil are observed. Therefore clarity of the solution is not proof of completion of the reaction.

Unsaponifiable matter is not removed and, if it is present in substantial amounts, it can interfere with the subsequent analysis. If this is the case, the method specified shall be supplemented with the following operations.

Dilute with water (4.4.3.1) the solution obtained after saponification and extract the unsaponifiable matter with diethyl ether, hexane or light petroleum. Acidify the aqueous solution and extract the fatty acids with isooctane (4.4.3.4) or hexane (4.4.3.8). Prepare the methyl esters from these as described in 4.4.5.3.

Add the appropriate amount (see Table 1) of the methanolic boron trifluoride solution (4.4.3.3) through the top of the condenser.

For fatty acids and soaps, introduce the test portion chosen from Table 1 into the appropriate flask. Add the appropriate amount (see Table 1) of the methanolic boron trifluoride solution into the flask. Fit the condenser to the flask.

Table 1 — BF_3 reaction conditions and reagents selection guide

Purpose	Test portion mg	Flask (4.4.4.1) ml	NaOH solution (4.4.3.2) ml	BF_3 solution (4.4.3.3) ml	Isooctane (4.4.3.4) ml
GLC	100 to 250	50	4	5	1 to 3
	250 to 500	50	6	7	2 to 5
IR/TLC	500 to 750	100	8	9	4 to 8
	750 to 1 000	100	10	12	7 to 10

4.4.5.3 Preparation of the methyl esters in isooctane solution

Continue boiling for 3 min. For oils with long-chain fatty acids, such as fish oils, continue boiling for 30 min.

Add the appropriate amount (see Table 1) of isooctane (4.4.3.4) to the boiling mixture through the top of the condenser.

Remove the flask from the heat source and remove the reflux condenser. Immediately, without allowing the flask to cool, add 20 ml of sodium chloride solution (4.4.3.5). Stopper the flask and shake it vigorously for least 15 s.

Add more of the saturated sodium chloride solution to bring the liquid level of the mixture into the neck of the flask. Allow the two phases to separate.

Transfer 1 ml to 2 ml of the upper isooctane layer into a 4 ml vial (4.4.4.5) and add a small amount of anhydrous sodium sulfate (4.4.3.6) to remove any traces of water.

The isooctane solution thus obtained is suitable for analysis using GC according to ISO 12966-4 as follows:

a) directly on to a packed column for gas-liquid chromatography;

b) after appropriate dilution with isooctane for capillary column systems prior to the injection;

c) after dilution with a lower boiling solvent such as isooctane for the special case of capillary on-column injection.

4.4.5.4 Storage of methyl ester solutions

The esters should preferably be analysed as soon as possible. If necessary, the isooctane solution containing the methyl esters may be stored under inert gas in a refrigerator. For a longer period of storage, it is advisable to protect the methyl esters against autoxidation by adding an antioxidant to the solution at a concentration that does not interfere with the subsequent analysis, e.g. a 0,05 g/l solution of 2,6-di-*t*-butyl-4-methylphenol (BHT). Methyl esters containing methyl butyrate shall only be stored in sealed ampoules, and special precautions shall be taken to prevent any loss by evaporation during filling and sealing of the ampoules.

4.4.5.5 Storage of dry methyl esters

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

NOTE 1 During GC of the methyl esters, certain reagents can produce unexpected peaks on the graph. Particularly during long storage, methanolic boron trifluoride can generate components which interfere in the C_{20} to C_{22} acids region.

NOTE 2 The effectiveness of derivatization can be determined by TLC as described in Annex A.

4.5 Acid-catalysed transmethylation of glycerides

4.5.1 Principle

The acidic methylation reagent causes transmethylation of glyceryl esters to fatty acid methyl esters. The methylation reagent also converts free fatty acids to fatty acid methyl esters.

4.5.2 Reagents

Use only reagents of recognized analytical grade, unless otherwise specified, and distilled or demineralized water or water of equivalent purity.

4.5.2.1 Sulfuric acid, $\rho_{20}(H_2SO_4) = 1{,}84$ g/ml.

4.5.2.2 Methanol, containing not more than 0,05 % mass fraction water.

4.5.2.3 Methylation reagent.

While cooling, add (4.5.3.2) slowly 1 ml sulfuric acid (4.5.2.1) to 15 ml (4.5.3.5) methanol (4.5.2.2).

4.5.3 Apparatus

Usual laboratory equipment and, in particular, the following.

4.5.3.1 Analytical balance, capable of being read to the nearest 10 mg.

4.5.3.2 **Graduated pipettes**, capacities 1 ml and 5 ml, ISO 835[1].

4.5.3.3 **Glass ampoules**, of capacity 5 ml.

Instead of glass ampoules, another suitable derivatization vial of similar dimensions may be used, provided that a leak-tight closure prevents losses of volatile esters.

4.5.3.4 **Oven**, capable of being maintained between 100 °C and 110 °C, or **boiling water bath**.

4.5.3.5 **Pipette**, 15 ml.

4.5.4 Preparation of test sample

Prepare the test sample as specified in 4.1.

4.5.5 Procedure

Weigh (4.5.3.1), to the nearest 10 mg, 100 mg of the prepared sample into a glass ampoule (4.5.3.3). Add 0,4 ml of methylation reagent (4.5.2.3). Flame seal the glass ampoule. Heat the glass ampoule and its contents for 3 h in the oven (4.5.3.4) maintained between 100 °C and 110 °C or on a boiling water bath (4.5.3.4), with intermittent mixing of the ampoule contents.

Cool the glass ampoule and its contents to room temperature and allow for complete phase separation. Open the glass ampoule and take an aliquot of the upper phase for further analysis. Dilution with an appropriate solvent of the esters formed can be necessary.

The final solution is suitable for analysis using GC according to ISO 12966-4.

NOTE The effectiveness of derivatization using the "general" procedure can be determined by TLC as described in Annex A.

Annex A
(informative)

Thin-layer chromatography method for testing the completeness of derivatization

A.1 Scope

This annex describes a thin-layer chromatography (TLC) method for the verification of whether derivatization performed according to 4.2, 4.3, 4.4 or 4.5 has occurred quantitatively. Additionally, this procedure can be useful in determining the generic composition of an oil or fat before any derivatization processes are undertaken.

A.2 Principle

TLC is used to resolve derivatization products or the generic constituents of fats and oils before derivatization is undertaken. Products are identified following either molybdophosphoric acid visualization or iodine vapour visualization by comparing the visualized product bands with those of test substances run on the same plate.

When derivatization is incomplete, unconverted reaction products, such as mono-, di-, and triglycerides as well as free fatty acids, are still identifiable in very low concentrations.

A.3 Reagents

A.3.1 Ready-made TLC/HPTLC plates or **aluminium sheets**, different sizes (e.g. 10 cm × 10 cm, 20 cm × 10 cm, 20 cm × 20 cm), coated with silica gel 60, layer thickness 0,2 mm to 0,25 mm. These plates may be cut in half to provide plates 50 mm × 100 mm.

A.3.2 Petroleum ether, boiling range 40 °C to 60 °C.

A.3.3 Diethyl ether, peroxide-free.

A.3.4 Glacial acetic acid, mass fraction 100 %.

A.3.5 Molybdophosphoric acid.

A.3.6 Ethanol, volume fraction 95 % to 96 %.

A.3.7 Molybdophosphoric acid solution, molybdophosphoric acid (A.3.5) in ethanol (A.3.6), volume fraction 3,5 %.

A.3.8 Iodine crystals.

A.3.9 Mobile phase, mixture of petroleum ether (A.3.2) (volume fraction 85 ml/101,5 ml) and diethyl ether (A.3.3) (volume fraction 15 ml/101,5 ml) and acetic acid (A.3.4) (volume fraction 1,5 ml/101,5 ml).

A.3.10 Test substances, e.g. free fatty acids, mono-, di-, and triglycerides as well as fatty acid methyl esters and propyl esters.

A.3.11 Potassium permanganate.