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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*

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**Contents**

Page

Foreword .....	iv
Introduction.....	v
1 Scope .....	1
2 Normative references .....	1
3 Reactions .....	1
4 Methodology .....	2
4.1 Preparation of test sample .....	2
4.2 Rapid method.....	2
4.3 General method .....	4
4.4 Transmethylation using boron trifluoride (BF <sub>3</sub> ) catalyst .....	6
4.5 Acid-catalysed transmethylation of glycerides.....	9
Annex A (informative) Thin-layer chromatography method for testing the completeness of derivatization .....	11
Bibliography.....	15

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## Foreword

ISO (the International Organization for Standardization) is a worldwide federation of national standards bodies (ISO member bodies). The work of preparing International Standards is normally carried out through ISO technical committees. Each member body interested in a subject for which a technical committee has been established has the right to be represented on that committee. International organizations, governmental and non-governmental, in liaison with ISO, also take part in the work. ISO collaborates closely with the International Electrotechnical Commission (IEC) on all matters of electrotechnical standardization.

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<sub>3</sub>) 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<sub>3</sub>) transmethylation/methylation

Owing to the toxicity of BF<sub>3</sub> it is recommended that this method only be used *in extremis*.

The BF<sub>3</sub> 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.

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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 transmethyrate 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.

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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

### 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  $\text{BF}_3$  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  $\leq 0,5$  % 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  $\text{BF}_3$ , it is recommended that the  $\text{BF}_3$  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<sup>[8]</sup>, 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<sup>[3]</sup>, 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<sup>[6]</sup>.

**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<sup>[2]</sup> 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.