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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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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.

The procedures used to develop this document and those intended for its further maintenance are described in the ISO/IEC Directives, Part 1. In particular the different approval criteria needed for the different types of ISO documents should be noted. This document was drafted in accordance with the editorial rules of the ISO/IEC Directives, Part 2 (see www.iso.org/directives).

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. Details of any patent rights identified during the development of the document will be in the Introduction and/or on the ISO list of patent declarations received (see www.iso.org/patents).

Any trade name used in this document is information given for the convenience of users and does not constitute an endorsement.

For an explanation on the voluntary nature of standards, the meaning of ISO specific terms and expressions related to conformity assessment, as well as information about ISO's adherence to the World Trade Organization (WTO) principles in the Technical Barriers to Trade (TBT) see the following URL: www.iso.org/iso/foreword.html.

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

This second edition cancels and replaces the first edition (ISO 12966-2:2011), of which it constitutes a minor revision.

The changes compared to the previous edition are as follows:

— milk and milk fat products have been excluded from the scope.

A list of all the parts in the ISO 12966 series can be found on the ISO website.

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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 of 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 document 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 the following:

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.

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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 can 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 can produce derivatives of fatty acids containing labile groups which can 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.

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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 document 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 document 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).

Milk and milk products (or fat coming from milk and milk products) are excluded from the scope of this document.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements 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 Terms and definitions

No terms and definitions are listed in this document.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— IEC Electropedia: available at http://www.electropedia.org/

— ISO Online browsing platform: available at http://www.iso.org/obp

4 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 (5.2) 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

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